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REVIEW ARTICLE

Molecular characterization of opportunistic pathogenic zygomycetes

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ABSTRACT The term zygomycosis refers to a diverse group of mycotic diseases caused by members of the orders Mucorales and Entomophthorales. These infections are frequently associated with diabetic ketoacidosis, deferoxamine treatment, cancer and its therapy, solid organ or bone marrow transplantations, extreme malnutrition and neutropenia. Although these mycoses are relatively rare, their high mortality rate underline the importance of this group of fungal infections. Molecular techniques are widely used to identify the virulence factors of clinically important fungi or to develop useful diagnostic techniques. However, application of these methods to characterize the opportunistic pathogenic nature of zygomycetes started only a few years ago. This review discusses the current state of molecular studies performed on the pathogenicity and diagnosis of zygomycetes causing opportunistic human mycosis.
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KEY WORDS

zygomycosis
molecular diagnosis
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genetic transformation
virulence factor

Zygomycetes fungi are characterized by the presence of aseptate wide hyphae (coenocytic mycelia) and the formation of zygospores. They are saprophytic filamentous fungi which are ubiquitous in soil and decaying organic materials. Several species belonging in the orders Mucorales and Entomophthorales have been reported to be agents of opportunistic human mycoses, designated as zygomycosis (mucormycosis).

Within the Mucorales, *Rhizopus* seems to be most frequently involved in zygomycoses, but *Absidia*, *Rhizomucor*, *Mucor*, *Apophysomyces*, *Saksanea*, *Cunninghamella*, *Cokeromyces* and *Syncephalastrum* species have also been isolated from clinical specimens (Ribes et al. 2000; Eucker et al. 2001; Freifeld and Iwen 2004); in fact, non-*Rhizopus* species are being increasingly recognized as causative agents of opportunistic mycoses (Walsh and Groll 1999). Healthy humans are generally unaffected, but those with weakened immunity are at risk of infection. The major risk factors for the development of zygomycosis are diabetic ketoacidosis; deferoxamine treatment to manage an iron or aluminium overload; cancer and its therapy; solid organ or bone marrow transplantations; prolonged steroid use; and extreme malnutrition or neutropenia (Ribes et al. 2000; Nucci 2003; Walsh et al. 2004). The spores produced by these fungi are airborne and can be inhaled into the respiratory tract: this is the most common route for the infection of a susceptible host. Zygomycoses manifest primarily as rhinocerebral infections, but pulmonary and disseminated mycoses also occur, as do gastrointestinal and subcutaneous infections and allergic diseases. 70% of rhinocerebral infections, which account for from one-third to one-half of all zygomycoses, are associated with diabetic ketoacidosis (Ribes et al. 2000). Rhinocerebral disease has a high mortality rate, but curation is possible (depending on the state of the patient) if an early diagnosis is followed by aggressive surgical and antifungal treatment.

Conidiobolus and *Basidiobolus* species, from the order Enthomophthorales, generally cause subcutaneous and mucocutaneous infections in tropical and subtropical areas.

The diagnosis of zygomycoses is very challenging: most such infections are identified only as zygomycosis or mucormycosis, without species or at least genus determination (Ribes et al. 2000; Eucker et al. 2001; Freifeld and Iwen 2004). Currently, the definitive diagnosis of zygomycosis is achieved by biopsy and histological study of the tissue lesions (Freifeld and Iwen 2004; Walsh et al. 2004). Culturing of an isolate from a tissue sample could be of help for the species identification. However, this is often difficult, because hyphal elements may be rare in tissue specimens and they can lose their viability during the tissue homogenization prior to culturing. Whenever an isolate is successfully isolated from a clinical sample, it is very important to maintain it for further analysis. Simple strain maintenance methods are well established for the zygomycetes (Palágyi et al. 1997) and can be easily carried out in clinical laboratories. Attempts have been made to elaborate diagnostic methods based on molecular and antigen detection techniques (Jones and Kaufman 1978; Hessian and Smith 1982; Pierce et al. 1982; Yankey and Abraham 1983; Kaufman et al. 1989; Zeilander et al. 1990; Voigt et al. 1999; Wu et al. 2003), but all of them are still in the experimental phase and are not yet used in clinical practice (Ribes et al. 2000).

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Although high doses are needed for successful therapy, amphotericin B is the classical antifungal agent of choice for the treatment of zygomycosis. Currently available triazoles have proved to be inactive as sole antifungal agent against zygomycetes (Garas et al. 1999; Tawara et al. 2000; Sun et al. 2002a). An exception is posaconazole, which exhibits activity *in vitro*, in animal models and in some patients (Sun et al. 2002b; Tobon et al. 2003). Applications of drug combinations for this fungal group are weakly documented. Surgical intervention is a standard part of the treatment of localized infections.

The high mortality rate (75-95%, depending on the form of the zygomycosis) and the fact that these fungi display intrinsic resistance to the most widely used antifungal drugs underline the importance of this group of fungal infections (Ribes et al. 2000; Eucker et al. 2001).

In the past decade, advances in fungal biology have made molecular techniques essential in the study of medically important fungi. These methods are used to identify their virulence factors, to determine new therapeutic targets or to develop useful diagnostic techniques. Although molecular methods are relatively well established for the zygomycetes, and especially for the genus *Mucor*, application of these techniques to study the opportunistic pathogenic nature of these fungi started only a few years ago. We review here the current state of pathogenic and diagnostic studies on clinically important zygomycetes, based on molecular genetic approaches.

Molecular taxonomy and strain typing

Techniques used in taxonomic studies and genotyping, such as PCR with species-specific primers, hybridizations with specific probes, mitochondrial and genomic restriction fragment length polymorphism (RFLP), sequence analysis of the ribosomal DNA and/or the internal transcribed spacer (ITS) region, single-strand conformational polymorphism (SSCP), random amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE), could serve as the basis for the development of rapid and specific DNA-based methods for the clinical diagnosis; moreover, molecular genotyping could reveal important data concerning the epidemiology of these infections.

Ribosomal DNA (rDNA) and some other gene sequences (such as actin and elongation factor 1) have been successfully used to reveal the phylogenetic relationships within the orders Mucorales (Voigt et al. 1999; O'Donnell et al. 2001; Voigt and Wöstemeyer 2001; Papp et al. 2003a) and Entomophthorales (Jensen et al. 1998). The results of these studies suggest that zygomycete systematics based primarily on morphological characteristics is highly artificial and a complete revision appears indicated (Nagy et al. 2004b). A number of markers have been identified that are potentially useful for further studies aimed at the development of diagnostic methods.

In contrast with the detailed phylogenetic studies on higher taxonomic levels, intraspecific genetic polymorphisms have been analyzed only relatively rarely in the zygomycetes. The taxonomic positions of several *Rhizomucor* strains have been determined and evaluated by ITS-RFLP (Vastag et al. 1998a; Vágvölgyi et al. 1999). On this basis, isolates were clearly demonstrated to be members of either *R. miehei* or *R. pusillus;* surprisingly, the single isolate of *R. tauricus* was identified as a mutant heterothallic *R. pusillus* strain. ITS-RFLP also distinguished *Apophysomyces elegans*, an emerging agent of zygomycosis with an increasing number of cases in India, from other clinically important zygomycetes, but the method was not able to demonstrate intraspecific polymorphism (Chakrabarti et al. 2003); microsatellite PCR fingerprinting was used to resolve this problem. ITS-RFLP and PCR fingerprinting allowed differentiation among the isolates of *Cunninghamella echinulata* and *C. bertholletiae* (Lemmer et al. 2002).

Among the varoius RFLP techniques, restriction patterns of mtDNA are frequently used in fungal taxonomy and genotyping, because this sensitive method indicates differences at a specific and even an intraspecific level. However, in the Zygomycetes, only the mtDNA organization of individual isolates of a few species have been reported, i.e. *M. racemosus* (Schramke and Orlowski 1993), *M. piriformis* (Papp et al. 1999), *R. stolonifer* (Paquin et al. 1997), *R. oryzae*, *Mortierella verticillata* and *Smittium culisetae* (Seif et al. 2005).

PFGE is also a versatile tool for molecular typing and to reveal the genetic variability at species and intraspecies levels. In the past 15 years, karyotypes of several zygomycetes, such as *Mucor circinelloides* (Vágvölgyi and Manczinger 1990; Nagy et al. 1994; Diaz-Minguez et al. 1999), *M. bainieri*, *M. mucedo*, *M. plumbeus*, *M. racemosus* (Nagy et al. 2000), *Parasitella parasitica* (Burmester and Wöstemeyer 1994) and species belonging in the genus *Micromucor* (Nagy et al. 2004) have been established. However, only a few data concerning opportunistic zygomycetes have been reported to date. The karyotypes of *Absidia glauca* strains have been revealed by rotating field gel electrophoresis (Kayser and Wöstemeyer 1991). Isolates with different mating types exhibited considerable differences in their electrophoretic karyotypes; a similar situation was observed in *M. circinelloides* (Diaz-Minguez et al. 1999).

RAPD analysis is also able to provide reproducible markers for strain identification. This method was previously used to establish specific PCR products able to differentiate between strains of several non-pathogenic zygomycete species, such as *P. parasitica* (Burmester and Wöstemeyer 1994), *M. piriformis* (Papp et al. 1997), *M. genevensis* (Vágvölgyi et al. 2001) or *Gilbertella persicaria* (Papp et al. 2001). RAPD analysis of *Rhizomucor* strains showed *R. miehei* to be genetically more homogeneous than the diverse *R. pusillus* (Vastag et al. 1999, 2000). These results strongly supported the observations of the earlier studies based on isoenzyme

analysis and carbon source assimilation assay (Vastag et al. 1997, 1998b). RAPD and the isoenzyme markers described in these works could be utilized in further studies to identify clinical and environmental isolates of *R. miehei* and *R. pusillus* and to check the accuracy of the original species identifications (Lukács et al. 2004b; Papp et al. 2004). The intraspecific variability of *Rhizopus stolonifer* and *R. oryzae* species was also examined by the RAPD method (Vágvölgyi et al. 2004a). Although only a few *R. oryzae* strains were involved in that study, the RAPD analysis appeared to support the unity of the species *R. oryzae*, which was established with the incorporation of about 30 strains originally described as independent species.

Molecular diagnostics

All types of infections caused by the zygomycetes are notorious as being difficult to diagnose and treat. Diagnosis in an early phase of the infection is essential for a successful outcome of zygomycoses. Recent attempts of diagnosis, based on the molecular and serologic techniques, are still in the experimental phase. In an early effort to apply molecular methods for diagnosis, 18S rDNA sequences with SSCP patterns were established to distinguish *Rhizopus* infections from those caused by other fungi (Walsh et al. 1995). Strains of *Rhizopus*, *Rhizomucor*, *Cunninghamella*, *Zygorhynchus* and *Mucor*, together with several clinically important non-zygomycete species, were involved in the development of a broad-range PCR assay (Van Burik et al. 1998). This method was optimized to detect fungal rDNA directly in the blood of patients, using long PCR probes. Although this non-specific assay is able to amplify the marker sequences of a broad range of fungal species, *Rhizomucor* and *Cunninghamella* proved to be non-detectable with the primers constructed in that study. The first significant advance in the development of a DNA-based identification method was the determination of 18S and 28S rDNA sequences from 42 zygomycetes involving the most common opportunistic pathogenic species (Voigt et al. 1999). Those authors designed 13 taxon-specific PCR primer pairs based on the 28S rDNA sequences to resolve the clinically most important zygomycetes, including different species of *Mucor*, *Rhizopus*, *Rhizomucor*, *Absidia*, *Cokeromyces*, *Cunninghamella*, *Basidiobolus* and *Conidiobolus*. Later, Wu et al. (2003) constructed a set of hybridization probes to detect the 18S rDNA of *Mucor* and *Rhizopus* species. Clinical strains of *Mucor*, *Rhizopus*, *Rhizomucor* and *Cokeromyces* were recently involved in an *in vitro* test of the commercially available MicroSeq D2 large subunit rDNA fungal sequencing kit (Applied Biosystems), which proved to be useful for the detection of these fungi (Hall et al. 2004).

Genetic transformation systems

The approaches most commonly used to examine the genetic background of pathogenic fungi are gene isolation, and the analysis of gene expression and gene disruption. The availability of an effective transformation system is a basic requirement for this type of molecular study.

The genetic transformation of a zygomycete fungus was first reported by van Heeswijck and Roncero (1984). They transformed a leucine auxotrophic strain of *M. circinelloides*, using a plasmid which harboured the α–isopropylmalate dehydrogenase gene (*leuA*). Their results have led to this species becoming a model organism for molecular studies on the class Zygomycetes during the past 20 years. Transformation procedures have additionally been elaborated for other zygomycetes, such as *A. glauca* (Wöstemeyer et al. 1987), *R. pusillus* (Wada et al. 1996, Yamazaki et al. 1999), *R. miehei* (Lukács et al. 2003, 2004a; Monfort et al. 2003; Vágvölgyi et al. 2004b), *Rhizopus niveus* (Yanai et al. 1990, 1991; Liou et al. 1992; Takaya et al. 1996), *R. delemar* (Horiuchi et al. 1995) and *R. oryzae* (Skory 2002, 2004; Michielse et al. 2003).

Mucor transformation protocols have traditionally been based on the $CaCl_2$/PEG-mediated methodology and necessitate protoplast formation from hyphae of young colonies or from germinating sporangiospores. The PEG-mediated transformation or electroporation of zygomycetes allows high-frequency transformations, but the introduced DNA remains almost exclusively extrachromosomal, replicating autonomously in the transformants (van Heeswijck and Roncero 1984; Revuelta and Jayaram 1986; Anaya and Roncero 1991; Iturriaga et al. 1992; Benito et al. 1995; Velayos et al. 1998; Wolf and Arnau 2002; Ács et al. 2003a; Papp et al. 2003b, 2005). In several cases, such transformants exhibit low mitotic stability. At times, integration in the genome can also be forced in these systems with the application of strong selection marker (Arnau et al. 1991; Arnau and Stroman 1993; Wada et al. 1996; Yamazaki et al. 1999) or the use of linear DNA fragments containing homologous flanking regions to drive the integration (Papp et al. 2002), but this is not the normal fate of the DNA introduced in these fungi. As a consequence, the genetic modification of the zygomycetes is generally hampered by the lack of an efficient integrative transformation system. To resolve this problem, the development of *Agrobacterium tumefaciens*-mediated transformation systems has been started for some zygomycetes, e.g. *R. oryzae* (Michielse et al. 2004), *R. miehei* (Monfort et al. 2003) and *M. circinelloides* (Nyilasi et al. 2003, 2005a), in order to achieve stable integrative transformant strains.

Most of the transformation systems involve auxotrophy complementation to select for the transformants. The most frequently used markers are the α–isopropylmalate dehydrogenase (*leuA* or *leu1*) and orotidine-5-monophosphate decarboxylase (*pyrG* or *pyr4*) genes, which complement leucine and uracil auxotrophy, respectively (van Heeswijck and Roncero 1984; Benito et al. 1995; Michielse et al. 2002; Wolf and Arnau 2002; Skory 2004). However, these selection

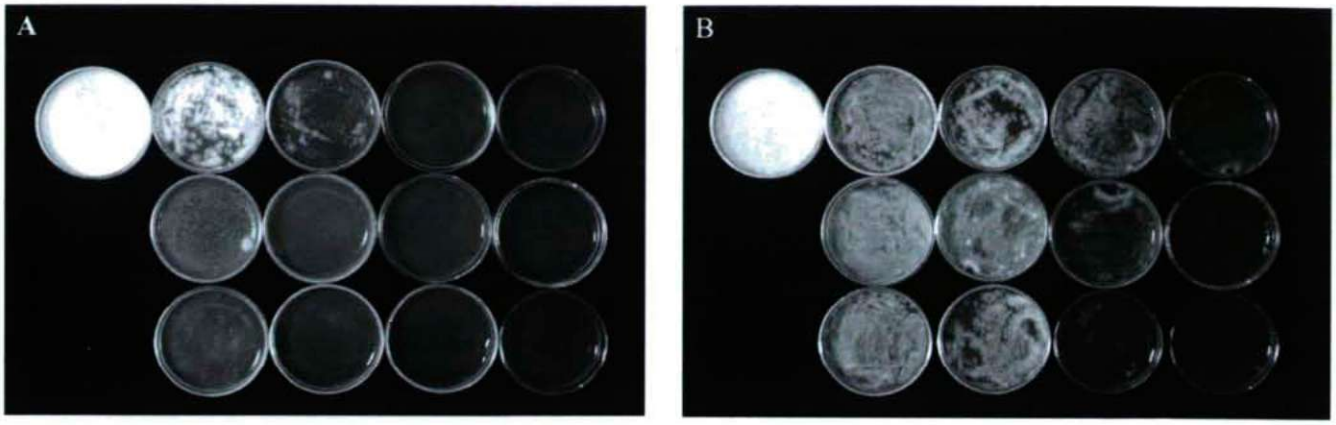

Figure 1. Effects of rose bengal and dichloran on the radial growth of *M. circinelloides*. A: colony formation of M20 on yeast extract-glucose (YEG) medium and YEG medium supplemented with 100 mg ml^{-1} hygromycin; 25, 50, 75, 100 mg ml^{-1} rose bengal (columns) and 1, 2, 3 mg ml^{-1} dichloran (rows). B: colony formation of transformant M20/A4 on YEG medium and YEG medium supplemented with 100 mg ml^{-1} hygromycin; 25, 50, 75, 100 mg ml^{-1} rose bengal and 1, 2, 3 mg ml^{-1} dichloran.

methods have the drawback that a stable auxotrophic mutant first has to be isolated from each strain that it is desired to transform. Dominant selection markers would make possible the direct transformation of wild-type strains. Unfortunately, most of the zygomycetes tested so far are highly resistant to various antibiotics. For example, *M. circinelloides* was reported to be resistant to hygromycin B, geneticin, nemoycin, oligomycin and benomyl (van Heeswijck et al. 1988). A method was recently developed for the hygromycin B-based selection of *Mucor* transformants: the sensitivity of the fungus was increased by the addition of rose bengal and dichloran to the culture medium (Fig. 1; Ács et al. 2003b; Nyilasi et al. 2005a).

To achieve heterologous gene expression in a host, it is usually necessary to combine the foreign gene with an adequate regulatory sequence of the host. Promoter sequences of the glyceraldehyde-3-phosphate dehydrogenase genes (*gpd*) have been widely used for the construction of expression vectors in different yeasts and filamentous fungi. These genes have also been isolated and characterized from a few zygomycetes: *M. circinelloides* (Ács et al. 2002; Wolf and Arnau 2002) and *R. miehei* (Ács et al. 2003c; Vastag et al. 2004). The 5' flanking region of *Mucor gpd1* has been applied as a strong and regulated promoter in an expression study (Larsen et al. 2004). With this same sequence, a dominant selection marker-based system was elaborated by Appel et al. (2004), who constructed a plasmid containing the Tn-5-derived kanamycin resistance gene combined with the promoter sequence of *Mucor gpd1*.

Identification of virulence factors

Thermotolerance, the production of efficient proteolytic, glycosidic and lipolytic extracellular enzymes (Ribes et al. 2000), siderophore production and an efficient iron transport system (Nyilasi et al. 2005b) have been suggested as the most likely virulence factors for opportunistic pathogenic zygomycetes. The isolation, cloning and disruption of a gene is an efficient means of analyzing the pathogenicity of a fungus and of determining and verifying possible virulence factors. Numerous genes encoding lipases, proteases and glycosidases have been isolated in the cases of *Mucor*, *Rhizopus*, *Rhizomucor* and other zygomycetes, but whether they play any role in the pathogenic processes remains unknown (Ribes et al. 2000). A high-affinity iron permease gene (*rFTR1*) of *R. oryzae* was recently cloned and studies to analyse its function were started by Fu et al. (2004). The transformation of the *rFTR* gene to *S. cerevisiae* partially restored the ability of an *ftr1* null mutant to grow on an iron-limited medium. Null mutant strains of *Candida albicans*, produced by gene disruption of the homologous *CaFTR* gene, previously displayed reduced virulence as compared with the wild-type strains in animal models (Ramanan et al. 2000). However, similar studies have not yet been reported with the *Rhizopus* gene. A homologous *FTR* gene of *R. miehei* and vector construction to produce null mutant strains for virulence studies were reported by Nyilasi et al. (2005c).

In the past year, sequencing of the genome of the *R. oryzae* clinical isolate RA99-880 was completed and the sequence assembly was published on the web by the *Rhizopus oryzae* Sequencing Project (Broad Institute of Harvard and MIT 2004; http://www.broad.mit.edu). An acceleration of molecular studies on the pathogenicity of the zygomycetes can be expected in consequence of this result.

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ARTICLE

Intranuclear differences in calmodulin gene expression in the trigeminal nuclei of the rat brain

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ABSTRACT The expression patterns of the three CaM genes were quantitatively localized by using in situ hybridization techniques to detect gene-specific [^{35}S]-labeled cRNA probes complementary to the multiple CaM mRNAs in the trigeminal nuclei of the adult rat brain. The three distinct CaM genes were widely expressed throughout the midbrain-brain stem area with moderate intensities. In general, mRNAs transcribed from the CaM III gene were the most abundant, followed by the CaM I and CaM II mRNA populations. Moreover, significant differences in the amounts of the transcripts of some CaM genes were found between the rostral and caudal parts of the individual nuclei of the trigeminal system. In most cases, the CaM gene-specific transcripts displayed a clear differential distribution along the rostrocaudal axis: they were more abundant in the rostral parts of these nuclei. For example, the levels of mRNAs transcribed from each of the CaM I, II and III genes were significantly higher in the rostral part of the principal sensory trigeminal nucleus, while the rostral part of the motor trigeminal nucleus exhibited an elevated amount of transcripts for the CaM I gene only. Interestingly, the CaM II mRNAs were most abundant in the caudal part of the mesencephalic trigeminal nucleus. Moreover, the largest difference between any of the CaM gene-specific transcript contents of the rostral and caudal parts was found for those of the CaM II gene in the principal sensory trigeminal nucleus. Here, the intranuclear difference was about 50%, the rostral part being the richer in CaM II mRNAs. Our results draw attention to the possible causal relation between the differences in the neuronal circuitry of the rostral and caudal parts of these nuclei and their differential CaM gene expression. This somatotopy may have important functional implications.
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Calmodulin (CaM), a multifunctional cytoplasmic calcium (Ca^{2+}) receptor protein encoded by three different genes in mammals, is especially abundant in the central nervous system (for reviews, see Palfi et al. 2002; Toutenhoofd and Strehler 2000). In our previous studies (some of them quantitative), we have already documented the regional distribution and expression pattern of the multiple CaM genes during normal development and in adulthood in the rat brain. The expression patterns corresponding to the three CaM genes displayed a widely differential distribution for the CaM gene-specific mRNA populations throughout the brain and spinal cord (Kortvely et al. 2002; Kovacs et al. 2002; Palfi et al. 1999, 2005).

As CaM exerts its biological action through its target proteins that are involved in a number of cellular regulator processes (see Kennedy 1989; Means et al. 1991; Palfi et al. 2002, for references), it is not surprising that immunohistochemical and *in situ* hybridization studies have demonstrated that CaM immunoreactivity or CaM gene-specific transcripts are often colocalized with those of the target enzymes of CaM within the same neuronal structures, not only in general, but in the brain stem-medulla region in particular (Erondu and Kennedy 1985; Ichikawa et al. 2004; Ochiishi et al. 1998; Ogawa et al. 2005; Seto-Oshima et al. 1983; Strack et al. 1996). In the brain stem-medulla area, however, only major neuronal structures have been quantitatively analyzed so far for the CaM gene expression (Palfi et al. 1999); the finer details are still unknown. In this respect, one of the unexplored areas concerns the trigeminal nuclei that form a complex sensory and motor system with precise somatotopic organization. The afferent components of this system carry various information from the skin of the face, the oral and nasal mucosa, and deeper structures such as subcutaneous tissues, facial muscles and tendons (Waite and Tracey 1995). The head also contains several specialized structures that receive trigeminal innervation, such as the teeth and tongue, the conjunctiva and cornea, and the vibrissae, which are under voluntary motor control (Dun 1958). For rodents, the arrangement, the structure and the innervation of the facial vibrissae are similar (Rice et al. 1986), and the pattern is determined genetically (Van der Loos et al. 1984).

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Since CaM plays an important role in regulating a key target protein, Ca/CaM kinase II, in a number of neuronal functions related to the trigeminal system, including inflammation, neuropathic pain and nerve injury (Liu and Simon 2003; Ogawa et al. 2005; Price et al. 2005), a precise, high-resolution mapping of the CaM gene expression could promote a deeper understanding of the functioning of the trigeminal system in health and disease. In our present study, involving quantitative *in situ* hybridization analysis through the use of CaM gene-specific [^{35}S]-labeled cRNA probes, we now report on the differences in CaM gene expression patterns seen along the rostrocaudal axis within each nucleus of the trigeminal system.

Materials and Methods

Experimental animals and tissue preparation

The experimental procedures were carried out in strict compliance with the European Communities Council Directive (86/609/EEC), and followed the Hungarian legislation requirements (XXVIII/1998 and 243/1998) regarding the care and use of laboratory animals. Five adult (200-220 g) male Sprague-Dawley rats were maintained under standard housing conditions and kept on a normal diet and tap water *ad libitum*. The animals were ether-anesthetized and decapitated between 1.00 and 3.00 p.m. The brain was quickly removed, and the brain stem-medulla area was separated, frozen and serially sectioned in a cryostat (20 μm) onto 3-aminopropyltriethoxy silane-coated glass slides and kept at -70°C until further processing (not longer than two days). The sections used in this study were cut from bregma -6.72 mm to -10.04 mm (Paxinos and Watson 1997), encompassing the entire area where the trigeminal system (mesencephalic, motor and principal sensory trigeminal nuclei) resides (see Fig. 1 for details). The spinal trigeminal nucleus was not included in this survey as its CaM gene expression pattern was weak, and did not allow an appropriate demarcation of this nucleus from other structures.

cRNA probes

Briefly, genomic sequences of the 3'-nonhomolog regions of CaM I, II and III mRNAs were amplified by polymerase chain reactions (PCRs) as described previously (Palfi et al. 1998); sequence alignment was completed with the software BLASTN version 2.0.6 (Zhang and Madden 1997). PCRs were performed by employing EcoR I and BamH I restriction enzyme cleavage site-extended primers. The primer sequences complementary to rat genomic DNA were as follows: for CaM I, 5'-AGACCTACTTTCAACTACT, corresponding to the 30-48-bp sequence, and 5'-TGTAAAACTCATGTAGGGG, corresponding to the 236-254-bp sequence of exon 6 (Nojima and Sokabe 1987); for CaM II, 5'-ATTAGGACTCCATTCCTCC, corresponding to the 144-162-bp sequence (numbered 1929-1947), and 5'-CACAACTCCACACTTCAACAGC, corresponding to the 353-374-bp sequence (numbered 2138-2159) of exon 5 (Nojima 1989); and for CaM III, 5'-ATGATGACTGCGAAGTGAAG, corresponding to the 12-31-bp sequence (numbered 7058-7077) of exon 6, and 5'-CAGGAGGAAGGAGAAAGAGC, corresponding to the non-transcribed genomic sequence 153-172-bp downstream to the stop codon (numbered 7228-7247; Nojima 1989). Standard PCRs were run for 35 cycles (Palfi et al. 1998), and the resulting PCR products were cloned into a pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA) and sequenced (AB 373 DNA Sequencer, PE Applied Biosystems, Foster City, CA, USA) to confirm their identity. *In vitro* RNA syntheses from the purified and linearized vectors were carried out to prepare antisense and sense cRNA probes. For radiolabeling, [^{35}S]UTPαS (1,100 mCi/nmol; Isotope Institute, Budapest, Hungary) was incorporated, using Riboprobe System-T7 and Riboprobe System-SP6 (Promega; Madison, WI, USA) according to the manufacturer's instructions. The complementary probe sequences were 225 bp (CaM I), 231 bp (CaM II) and 157 bp (CaM III) long. Labeled probes were purified by size exclusion chromatography. The probe-specific activities of the radiolabeled hybridizing sequences were determined to be 1.97-4.38 x 10^7 cpm/pmol. The specificity of the 3 antisense probes had previously been determined by sequence alignment, Northern blot analysis and *in situ* hybridization (Palfi et al. 1998, 1999).

In situ hybridization

Radioactive *in situ* hybridization was carried out according to previously published protocols (Palfi et al. 1998, 1999) Briefly, coronal cryostat medullary sections were fixed for 5 min in 2x SSC containing 4% formaldehyde, washed twice in 2x SSC for 1 min, and then rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride at room temperature (RT) for 5 min. The sections were dehydrated, air-dried and then hybridized in 100 μl hybridization solution (50% formamide, 5x SSPE, 1x Denhardt's reagent, 10% dextran sulfate, 50 mM DTT, 100 μg/ml salmon sperm DNA and 100 μg/ml yeast tRNA) containing 200 fmol/ml [^{35}S]-labeled riboprobe. The pH of the solution was adjusted to 7.4. Hybridization was performed under parafilm coverslips in a humidified chamber at 55°C for 20 h. The sections hybridized with [^{35}S]-labeled riboprobes were extensively rinsed in 2x SSC/50% formamide at 50°C, treated with 16 μg/ml RNase A at 37°C for 30 min, washed again several times in 2x SSC/50% formamide at 50°C, and then dehydrated, air-dried and processed for autoradiography.

Autoradiography and image analysis

Tissue sections hybridized with radioactive riboprobes were exposed to Kodak BioMax MR-1 films (Eastman Kodak Co., Rochester, NY, USA) for 5 days at -20°C and developed. At

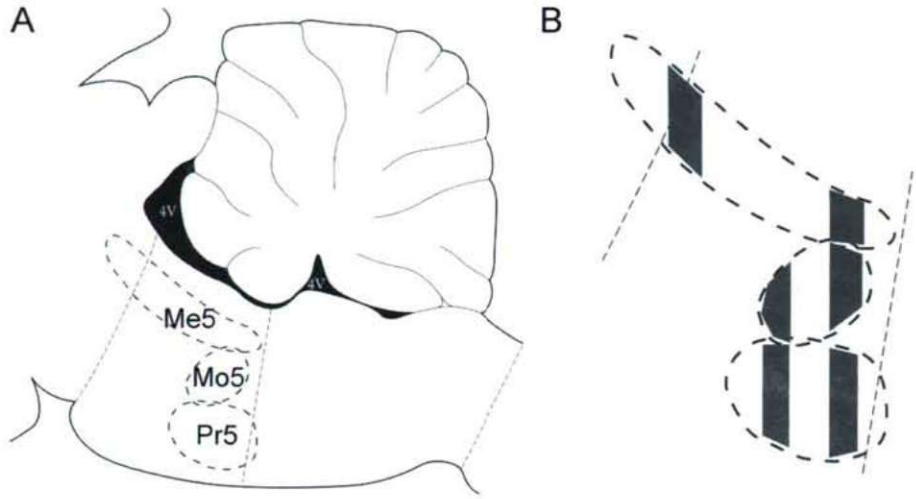

Figure 1.A) Schematic representation of the trigeminal nuclei (ovals outlined with dashed lines) in a section of the rat brain cut in a sagittal plane. Me5: mesencephalic trigeminal nuclei, Mo5: motor trigeminal nuclei, Pr5: principal sensory trigeminal nucleus, 4V: fourth ventricle. Note that these nuclei reside in different sagittal planes, and are never seen together as in this scheme (see Figure 2 A and B). B) Enlarged schematic view of the trigeminal nuclei. The tissue sections (vertical areas shaded light gray) used for analysis in this study were cut from bregma -7.64 to -7.80 for the rostral, and -9.30 to -9.68 for the caudal part of Me5, from bregma -8.80 to -9.16 for the rostral, and -9.30 to -9.68 for the caudal part of Pr5, and from bregma -8.80 to -9.16 for the rostral, and -9.30 to -9.68 for the caudal part of Mo5 (coordinates according to Paxinos and Watson (1997)). The portion of Me5 that resides more rostral than indicated here is not suitable for the measurement of CaM gene expression because the cells cannot be grouped together with certainty.

least three separate hybridization experiments for each animal were carried out for each CaM gene, and the gray-scale values for each CaM gene were measured in at least three consecutive sections. Autoradiographic images of hybridized sections were scanned at 600 x 600 dpi resolution and analyzed by the computer program Image J (version 1.32; developed at the U.S. National Institutes of Health by W. Rasband, and available from the Internet at http://rsb.info.nih.gov/ij). Regions of interests within the trigeminal system were outlined on the computer screen and their signal intensities were measured. It is important to note that some parts of the trigeminal nuclei cannot be delineated properly on the basis of the autoradiographic distribution of their CaM gene-specific mRNA contents. Thus, for example, we were unable to discriminate between certain parts of the principal sensory trigeminal nucleus; accordingly, gray-scale values for its dorsomedial and ventrolateral parts are not reported separately here. Gray values between 0 (lightest) and 255 (darkest) were assigned to the images, and the specific gray values were determined by subtracting the sense values from the corresponding antisense values. Analysis of significance was carried out with the two-tailed Student's *t*-test (Microsoft Excel 2004 for Mac, Ver. 11.2; Microsoft, Redmond, WA, USA).

Results

In situ hybridization of [^{35}S]-labeled antisense CaM I, II and III cRNA probes to tissue sections of the brain stem-medulla region established a specific and unique distribution of the autoradiographic label (Fig. 2), whereas hybridization with a sense probe (not shown) resulted in a very low labeling with nonspecific distribution. CaM mRNAs transcribed from the three CaM genes were widely distributed, albeit generally with low-to-medium levels, throughout this brain area. Quantitative image analysis of the autoradiograms revealed that mRNAs transcribed from the CaM III gene were generally most abundant, followed by CaM I and CaM II mRNA populations (Table 1). This rank order of signal intensity is identical to that reported for other brain (Palfi et al. 1999) and spinal cord areas (Kovacs and Gulya 2002). The highest specific optical density was detected in the motor trigeminal nucleus for CaM III transcripts, and the smallest one in the principal sensory trigeminal nucleus for the CaM II gene. For some CaM genes, significant differences in the amounts of their transcripts were found between the rostral and caudal parts of the individual nuclei of the trigeminal system. In most cases, the CaM gene-specific transcripts were more abundant in the rostral parts of the nuclei, as the levels of mRNAs transcribed from each of the CaM I, II and III genes were significantly higher in the rostral part of the principal sensory trigeminal nucleus, while the rostral part of the motor trigeminal nucleus displayed an elevated amount of transcripts for the CaM I gene only. For example, the levels of CaM I transcripts were about 38% and 37% higher in the rostral parts of the principal sensory trigeminal nucleus and the motor trigeminal nucleus, respectively. Interestingly, the CaM II mRNAs were most abundant in the caudal part of

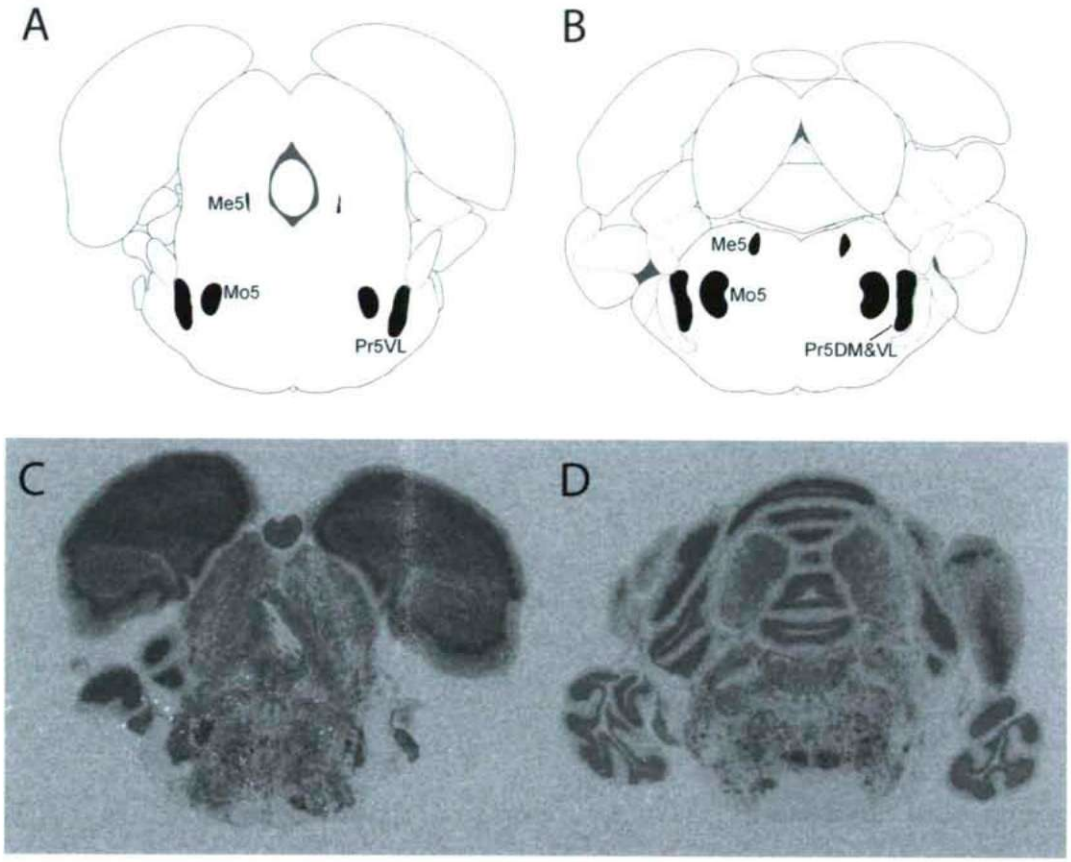

Figure 2. Differential CaM gene expression in the trigeminal nuclei of the rat brain, as evidenced by the specific hybridization of antisense CaM I-gene specific [^{35}S]cRNA probes. A, B) Diagrammatic representation of the medullary nuclei (areas labeled solid black) in the rat brain (Paxinos and Watson 1997). Me5: mesencephalic trigeminal nuclei, Mo5: motor trigeminal nuclei, Pr5VL: principal sensory trigeminal nucleus, ventrolateral part, Pr5DM&VL: principal sensory trigeminal nucleus, dorsomedial and ventrolateral parts. C, D) Specific hybridization of antisense CaM I-gene specific [^{35}S]cRNA probes to the coronal sections of the rat brain stem-medulla area. Representative pictures taken from bregma -8.80 (C) and -9.30 mm (D), respectively.

the mesencephalic trigeminal nucleus. The largest difference between any CaM gene-specific transcript contents of the rostral and caudal parts was found for the CaM II gene in the principal sensory trigeminal nucleus, where the levels of these transcripts were otherwise characteristically the lowest. The intranuclear difference here was about 50%, the rostral part being the richer in CaM II mRNAs.

Discussion

Among the cranial nerves that carry sensory information to the central nervous system, the rodent trigeminal system is of special interest because of its precise somatotopic organization (Waite 1984; Waite and Tracey 1995). The trigeminal sensory nuclei are divided into three groups: the mesencephalic nucleus (Me5), the principal sensory nucleus (Pr5), and the spinal trigeminal nucleus (Sp5), this latter being subdivided into the nucleus spinal subnuclei oralis (Sp5O), the nucleus interpolaris (Sp5I) and the nucleus caudalis (Sp5C). The organization of terminations in the main and spinal nuclei is most clearly evident for vibrissal afferents, for which a pattern analogous to the peripheral arrangement of vibrissae can be discerned in coronal sections. Each vibrissa is associated with a patch or barrel (for references, see Waite and Tracey 1995). In general, three representations of the vibrissae are seen, in Pr5, Sp5I and Sp5C; although Sp5O receives vibrissal terminations, no patches are evident in this subnucleus. In the horizontal plane, terminations from each vibrissa are seen as long rostrocaudal columns throughout the nuclei in a somatotopic pattern. The presence of the somatotopic pattern of the rostrocaudal barrels is most evident for the principal sensory nucleus. Our CaM expression data here could be interpreted as showing a differential CaM mRNA distribution along the rostrocaudal axis of this nucleus, which is a consequence of the separate information received from the rostral and caudal vibrissal fields.

The motor trigeminal nucleus of the rat is divided into a large dorsolateral division extending throughout the ros-

Table 1. Quantitative analysis of CaM gene expression in the trigeminal nuclei of the adult rat brain.

Component of the trigeminal system	Gene	Average specific gray-scale value (± S.D.) rostral	caudal	% of rostral value
Mesencephalic trigeminal nucleus	CaM I	63.28 ± 11.79	64.27 ± 6.95	98.46
	CaM II	56.12 ± 6.67	73.33 ± 8.09*	76.53
	CaM III	86.31 ± 19.64	60.21 ± 8.20	143.35
Principal sensory trigeminal nucleus	CaM I	76.78 ± 6.85	55.78 ± 9.33*	137.65
	CaM II	43.17 ± 7.72	28.86 ± 3.70*	149.58
	CaM III	80.87 ± 5.10	66.97 ± 6.69*	120.76
Motor trigeminal nucleus	CaM I	79.02 ± 7.48	57.48 ± 9.84*	137.47
	CaM II	38.62 ± 7.55	42.13 ± 6.23	91.67
	CaM III	88.22 ± 6.85	77.26 ± 8.06	114.19

Coronal cryostat sections from the pons-medulla area were cut, hybridized separately with antisense [^{35}S]cRNA probes specific for CaM I, CaM II or CaM III mRNAs and exposed to autoradiographic film. Film autoradiographic images (at least 4 in each of the 5 animals for a given structure) were analyzed by computer-assisted microdensitometry. Gray-scale levels for each CaM gene were determined by using the image analysis computer program Image J (ver. 1.32). High-resolution gray-scale images were taken and the brain stem structures of interest were outlined on the computer screen. Specific gray-scale values (means of at least 20 measurements from 5 separate experiments ± S.D.) were calculated by subtracting the nonspecific values resulting from the hybridization of the respective sense cRNA probes from the values of the antisense [^{35}S]cRNA probes. *Significant differences ($p < 0.05$, two-tailed Student's *t*-test) were found between the rostral and caudal parts of the trigeminal nuclei.

trocaudal length of the nucleus and a smaller ventromedial division in the caudal two-thirds (for references, see Travers 1995). The jaw closing muscles, the masseter, the temporalis and the medial pterygoid, are innervated from the dorsolateral division, while two jaw opening muscles, the anterior digastric and the mylohyoid, are innervated from the ventrolateral division. A third jaw opening muscle, the lateral pterygoid, is grouped together with jaw closing motoneurons in the ventral aspect of the dorsoventral division. Even though the areas corresponding to the dorsolateral and ventromedial divisions are not distinguishable on the basis of their CaM gene expression patterns in the coronal sections of the caudal part of the nucleus, the caudal part nevertheless clearly has higher levels of CaM mRNA populations.

The major organizing principles of the vertebrate central nervous system determine that its parts receive inputs from separate sources, while separate efferent connections project to different neuroanatomical entities. Previous studies have established that certain nuclei in the central nervous system display a differential structural and functional organization, mainly along their rostrocaudal axis, that can be seen in the differences in their neuronal circuitry, neurochemical cytoarchitecture or gene expression pattern. This somatotopy is also present in the brain stem, one of the nuclei most characteristically expressing this feature being the parabrachial nucleus (PBN). The PBN has been divided into at least 13 distinct subnuclei and regions, each associated with a unique set of afferents, efferents and neurotransmitters (for references, see Saper 1995), which frequently mark out distinct terminal fields according to their receptive fields. For example, projections to the PBN from the nucleus of the solitary tract carry afferent signals from both the oral cavity and the gastrointestinal tract. Although physiological studies have suggested the convergence of oral and gastrointestinal sensory signals in the PBN, anatomical studies have emphasized the segregation of these pathways in the rat. Karimnamazi et al. (2002) found that the gastric terminations in the PBN were separate from the taste projections in the rostral portion of the external lateral and dorsal lateral subnuclei, while the gustatory projections were separate from the gastric terminations in the ventral lateral and central medial subnuclei of the caudal "waist" region, and were intermingled with the gastric projections in these subnuclei and the external subnuclei at slightly more rostral levels. The physiological evidence for overlap in the PBN was also evaluated, as neurophysiological recordings demonstrated that a small proportion of single cells within the waist and external subnuclei could be activated by both gastric and orotactile stimulation. The behavioral roles of the „waist" area and external subnuclei of the PBN in the processing of gustatory information have also been defined by monitoring oromotor behavior in the areas within and surrounding this nucleus (Galvin et al. 2004). Electrical and chemical stimulation of the "waist" area increased ingestive oromotor behavior, while stimulation of the external parabrachial subnuclei and areas medial and ventral to the nucleus did not result in a behavioral change. These data supported the hypothesis that the waist area of the PBN constitutes part of the neural substrate involved in eliciting oromotor behavior in response to taste input. However, another experiment (Gulya et al. 1991) provided indirect evidence for the separation of functions within a nucleus that resides outside the brain stem. It was reported that the vasopressin-containing cells in the bed nucleus of the stria terminalis responded to dehydration and/or to ethanol treatment in a subregion-dependent manner within the nucleus. Dehydration affected only cells in the central (or medial) region, while ethanol ingestion also

affected cells in the caudal region of the nucleus. As the different parts of this nucleus send afferents both to the lateral septum and to the neurohypophysis in rodents (Kelly and Swanson 1980), or to several regions of the mesencephalon, pons and medulla oblongata in the cat (Holstege et al. 1985), the anatomical basis for the segregation of functions, involving the differential regulation of vasopressin gene expression, is plausible.

In summary, our results draw attention to a possible causal relation between the differences in afferent and efferent neuronal connections (and consequently in their presumably segregated functions) of the rostral and caudal parts of the trigeminal nuclei and their differential CaM gene expression.

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ARTICLE

High incidence of human papillomavirus infection in cervical carcinoma patients in South Hungary

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ABSTRACT The aim of this study was to determine the incidence of human papillomavirus (HPV) in cervical carcinoma patients. A nested case-control study was performed to investigate the relationship between HPV infection and cervical carcinoma. A total of 347 women 169 of whom gave abnormal Pap smear tests; were recruited to participate in the study; 39 of them suffered from invasive carcinoma. The overall incidence of HPV infection in the cancer, positive cytology and normal cytology groups was 74% (29/39), 55% (72/130) and 4% (7/178), respectively ($p<0.001$). The risk for progression to cervical carcinoma when the HPV infection was associated with abnormal cytology was 2.16 (95%, CI: [1.01-4.69]). The incidence of HPV infection associated with abnormal cytology correlated significantly with the presence of cervical carcinoma.
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KEY WORDS

HPV infection
cervix carcinoma
abnormal Pap smear
epidemiology

Infection of the uterine cervix with human papillomavirus (HPV) usually occurs via sexual transmission; it can lead to malignant transformation. It is now believed that at least 17 types of HPV are associated with cervical cancer. The oncogenic virus type can be identified in nearly all the cervical cancers. They are not only associated with, but are also thought to be causative, of the cancer. Various mechanisms that contribute to the development of HPV-induced cancer have been described. The multistep process from HPV infection to carcinogenesis is not yet completely understood. HPV genetic sequences have been observed to be integrated into the host genome just as the cell develops invasive properties (Cullen et al. 1991). The E6 protein produced by high-risk HPV types 16 and 18 is known to be able to combine with the p53 protein and to cause the same functional consequence as a p53 gene mutation (Scheffner et al. 1990; Hoppe-Seyler and Butz 1993). Immunosuppression can also give rise to an increased risk of cervical neoplasia. Immunosuppression has been found to be associated with an increased rate of HPV infection in several studies (Sillman et al. 1984; Vermund et al. 1991) and, in consequence of the deficient host-regulatory mechanisms, allows neoplastic proliferation (Rock et al. 2000). HPV types 16, 18, 33, 31, 53 and 58 are most commonly associated with cervical oncogenesis. However, there is considerable heterogeneity in the geographic distribution of oncogenic HPV.

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Previous studies have investigated the prevalence and risk factors of HPV infection in Hungary, but not in association with cervical carcinoma (Deák et al. 1999; Nyári et al. 2004).

The aim of this study was to determine the incidence of HPV in cervical carcinoma patients, in order to facilitate the prediction of the possibility of development of cervical cancer in certain groups (high-risk HPV-infected patients).

Materials and Methods

During the period between January 2002 and September 2003, a nested case-control study was performed to investigate the relationship between HPV infection and cervical carcinoma at the Department of Obstetrics and Gynaecology of the University of Szeged. Cervical samples were collected for cytology and HPV testing from women attending the gynaecological outpatient clinic. Colposcopic and routine gynaecological examinations were performed in each case. Both the Papanicolau (Pap) and Bethesda classifications were used for cytology evaluation. Sampling, sample transport and HPV DNA determination via HPV hybrid capture assay were carried out in accordance with the instructions of the manufacturer of the kit (DIGENE HPV hybrid capture 2). Virus types were classified into two categories: high-risk HPV (including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), and low-risk (6, 11, 42, 43 and 44) types.

Data concerning age, occupation, lifestyle and health status were extracted from the patient register system.

Table 1. Distribution of HPV infections according to subtypes.

Group	No. of cases				p value
	Negative	Low-risk types	High-risk types	Total	
Normal Pap smear	171	4	3	178	<0.001
Abnormal Pap smear without cervical carcinoma	58	60	12	130	
Cervical carcinoma	10	4	25	39	

Statistical analyses were carried out with the STATA software package. The statistical methods used were the chi-square test and analysis of variance. To obtain an overview of the risk, logistic regression analysis was performed. A probability level of $p < 0.05$ was considered statistically significant.

Results

A total of 347 women with a mean of age of 42.9 years (SD 9.5) were recruited into the case-control study: 178 of them gave normal Pap smear test (these women served as control group) and 169 women gave abnormal Pap smear test (class III or higher), 39 of them were diagnosed with invasive carcinoma. This later group immediately underwent appropriate treatment.

The overall incidence of HPV infection in the cancer, positive cytology and normal cytology groups was 74% (29/39), 55% (72/130) and 4% (7/178), respectively ($p<0.001$). High-risk HPV subtypes were diagnosed in 86% (25/29 cases) of the HPV-infected cancer cases, 16% (12/72) of the HPV-infected cases of those who gave abnormal Pap smear test. The distribution of the HPV subtypes is shown in Table 1 and the age-specific distribution of HPV infection in Table 2. There were 46 and 5 low-grade squamous intraepithelial lesions cases in the abnormal Pap smear group and the control group, respectively.

HPV infection significantly increased the risk of abnormal cytology (odds ratio (OR) 30.5 95% confidence interval (CI) [13.3-70]) and the risk of cervical carcinoma (OR 68.8 95%, CI [24.2-195.6]). Further, the OR for progression to cervical carcinoma when the HPV infection was associated with abnormal cytology was 2.16 (95%, CI: [1.01-4.69]).

We did not find any significant difference in the incidence of HPV as a function of the place of residence or the previous obstetrical history of the women.

Discussion

The present study focused on the relationship between the incidence of HPV infection and that of cervical cancer. HPV infection associated with abnormal cytology correlated significantly with the development of cervical carcinoma.

In the 39 cancer cases, we found a rate of HPV infection of 74% (29 cases), 25 of these patients were infected by high-risk HPV, *i.e.* the proportion of high-risk HPV among the HPV-infected cancer patients was very high (86%). The corresponding proportion among the HPV-infected non-cancer patients was only 16% (12/72). The odds ratio for the progression to cervical carcinoma when the HPV infection was associated with abnormal cytology was 2.16 which represents significantly increased risk to develop cervical carcinoma (Branca et al. 2003).

In the second half of the 1990s, HPV testing was generally

Table 2. Age-specific distribution of HPV infection (n=347).

Age	Control group				Abnormal Pap smear group			
	Number of HPV-negative cases	Number of HPV-infected cases	Total	% of infected cases	Number of HPV-negative csaes	Number of HPV-infected csaes	Total	% of infected cases
20-29 years	0	0	0		10	30	40	75%
30-39 years	41	1	42	2%	10	21	31	68%
40-49 years	94	6	100	6%	25	15	40	38%
50-59 years	36	0	36	0%	9	6	15	40%
60-69 years	0	0	0		4	0	4	0%
Total	171	7	178	4%	58	72	130	55%

Age	Cervix carcinoma group			
	Number of HPV-negative cases	Number of HPV-infected cases	Total	% of infected cases
20-29 years	0	0	0	
30-39 years	2	4	6	67%
40-49 years	3	12	15	80%
50-59 years	4	5	9	56%
60-69 years	1	8	9	89%
Total	10	29	39	74%

applied for the clinical screening of women of fertile age in Hungary. However, with regard to the results of international studies on large numbers of patients, and from cost-benefit considerations this practice was later modified (Schafer et al. 1991). We currently perform HPV testing only when this is suggested by the results of cytological examinations carried out because of the possibility of HPV infection.

The regular clinical screening of HPV-infected patients and their treatment by conization has effectively reduced the development of cervical cancer (Rock et al. 2000; Tachezy et al. 2003). One result of our study was a knowledge of the incidence of HPV infection in cervical cancer in Hungary, which had previously not been well documented.

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ARTICLE

Evaluation of toxicological implications of inhalation exposure to kerosene fumes and petrol fumes in rats

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ABSTRACT Toxicological implications of exposure to ungraded concentrations of kerosene and petrol fumes in albino Wistar rats were investigated after two weeks of 4 hours daily inhalation. Serum aminotransferases (AST and ALT), alkaline phosphatase (ALP), total cholesterol (Chol), triglyceride (TG) levels and histological analysis of the liver tissues were used as diagnostic markers to assess liver dysfunction. The mean levels of these markers determined for the group of rats exposed to kerosene and petrol fumes (test groups), as compared with the levels for the control group were significantly ($p < 0.05$) higher. ALT, AST and ALP levels of the kerosene exposed group were raised by 191%, 161% and 204% while serum total cholesterol and TG levels increased by 129% and 118%, respectively. The increases in the serum levels of AST, ALT, ALP, Chol, TG in the petrol exposed group were 177%, 140%, 191%, 100% and 97%, respectively, when compared with the controls. Histological analysis of the liver tissues of the experimental test groups indicated degenerative changes in the ultrastructural integrity of the hepatic cells. These results showed that frequent exposure to kerosene and petrol fumes may be highly deleterious to the liver cells. **Acta Biol Szeged 49(3-4):19-22 (2005)**

KEY WORDS

kerosene
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liver enzymes
histopathology
serum lipids

Kerosene and petrol are distilled from crude petroleum and vapours obtained from their evaporation may be considered as kerosene and petrol fumes. These fractions of crude petroleum contain aliphatic, aromatic and a variety of other branched saturated and unsaturated hydrocarbons (Henderson et al. 1993; Kato et al. 1993; Anderson et al. 1995). It has been demonstrated that after inhalation of equal concentrations of petroleum vapour through chronic exposure, lower concentrations of saturated hydrocarbons are detected in human and animal blood than that of the unsaturated aromatic hydrocarbons (Zahlsen et al. 1993). Biological monitoring of exposure to bitumen fumes during road-paving operations indicated urinary excretion of 1-hydroxypyrene and thioethers in the exposed workers (Burgaz et al. 1992).

Petroleum fumes are ubiquitous in our environment and the common sources of contact or exposure are petrochemical industries (refineries, oils fields, filling stations) and homes. The applications of kerosene as cooking and lighting fuels in the home have resulted in direct exposure of these products to a good percentage of the populace. Moreover, the day-to-day use of petrol outside the industrial settings is likely to have the same effect on the users as kerosene since they have been reported to contain most of the same hydrocarbons. However, the most affected are those who occupationally exposed to the fumes (Smith et al. 1993; Carballo et al. 1994; Rothman et al. 1996).

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Despite the high utilization of kerosene and petrol, our knowlegde is sparse on the toxicological effects of inhaling the composite fumes evaporating from kerosene and petrol. However, mutagenic, genotoxic, carcinogenic, neurotoxic, immunotoxic and haemotoxic effects of some petroleum and petrochemical products' constituents have been reported in experimental studies on humans and animals (Sim 1980; Hu and Wells 1994; Hallier et al. 1995). Hydrocarbons and other constituents of petroleum and petrochemical products, like other xenobiotics, are metabolized in the liver to a greater extent (Sims 1980; Nelson et al. 1993). Ueng et al. (1998) reported that exposure of rats to motorcycle exhaust and organic extracts of the exhaust particulate caused a dose- and time-dependent increase in cytochrome P-450-dependent monoxygenases and glutathione-S-transferase in the liver, kidney and lung microsomes. Since kerosene and petrol contain some of these constituents, chronic or frequent exposure to their fumes may affect the normal liver functions.

The expression of toxicity of xenobiotics is usually determined biochemically by the monitoring of some plasma enzymes and lipids. A rise in AST, ALT, ALP, TG and cholesterol are commonly measured as indices of the damage of the liver cells (Abdel-Baset et al. 1997; Owu et al. 1998). In this study we attempt to place on record the biotoxicity effects of kerosene and petrol fumes on albino Wistar rats by measuring some of these parameters. Histopathological changes in the liver tissues of both the control and experimental animals were also examined to support the biochemical findings.

Table 1. Effects of exposure to kerosene and petrol fumes on serum ALT, AST, ALP, Chol and TG levels of Wistar albino rats.

Group	ALT (U/L)	AST (UL)	AST/ALT	ALP (UL)	Chol (mmol/l)	TG (mmol/l)
Control	10.37±0.86	11.40±1.50	1.10	90.95±5.55	1.54±0.26	1.02±0.05
Kerosene fumes	30.12±0.58	29.72±1.37	0.99	276.24±3.36	3.53±0.33	2.22±0.05
Petrol fumes	28.70±1.06	27.33±3.06	0.95	264.67±0.36	3.08±0.36	2.01±0.09

Materials and Methods

Experimental animals

Young adult Wistar albino rats obtained from the animal house of the College of Medical Science, University of Calabar, Nigeria, were used for this study. Rats weighing 80-113 g were randomly selected into 3 groups (control, kerosene and petrol, respectively) of eight animals each. Kerosene and petrol were obtained from the Mobil Filling station, Calabar, Nigeria. All experimental animals were housed in stainless steel cages (60 cm x 30 x 45 cm) in a well-ventilated animal house and had free access to water, and normal rat chow obtained from Livestock Feeds, Calabar, Nigeria. The test groups were exposed to kerosene and petrol fumes, respectively, while the control group was kept in a section of the experimental animal house free from petroleum fumes.

Exposure to kerosene and petrol fumes

The method of exposure employed in this study was by inhalation. The animal cages housing the test groups were placed in exposure chambers measuring 150 cm x 90 cm x 210 cm. Two highly perforated 1000 ml cans containing 500 ml of kerosene were placed in the exposure chamber and the animals were allowed to inhale the fumes evaporating from the cans. The same procedure was adopted for the petrol fumes. In both cases, exposure lasted for 4 h daily for a period of 2 weeks. The time of exposure was 9.00 am to 1.00 pm, after which the animals were transferred to fumes-free section of the experimental animal house.

Collection of blood samples and liver tissues for analysis

The animals were anesthetized with chloroform 24 h after the last exposure and blood samples collected by cardiac puncture into plain sample tubes. Serum samples were separated 1 h after extraction of blood by centrifugation at 3000 g for 5 min and stored in a refrigerator. Biochemical analyses on the serum samples were done 24 h after sample collection. The liver tissues were collected and processed according to the method reported by Akpanabiatu et al. (2003).

Biochemical analyses were carried out for the measurement of serum alanine (ALT) and aspartate aminotransferases (AST), alkaline phosphatase (ALP), cholesterol (Chol) and triglyceride (TG) levels. Laboratory kit reagents (Randox laboratory Ltd, UK) were used for all biochemical analyses and the absorbances were read using a UV-Vis spectrophotometer (DREL 3000 HACH). Statistical analysis was carried out by employing Student's *t*-test to compare the mean values of the test groups with the controls ($p < 0.05$ was used as a level of significance).

Results and Discussion

The serum enzymes activities of the controls and the animals exposed to kerosene and petrol fumes are shown in Table 1. The activity of ALT was significantly higher in animals exposed to the kerosene and petrol fumes when compared to the controls. The level of serum ALT activity has been reported to be increased as a result of liver injury in patients developing severe hepatotoxicity (Beckett et al. 1989). ALT might have leaked from damaged cells, due to increased permeability of the hepatocellular membrane, or due to necrosis, indicating organ dysfunction (Mclntyre and Rosalki 1992).

The activity of AST was also significantly higher in the animals exposed to kerosene and petrol fumes when compared to the control animals. Increased activity of AST has been reported in CCl_4-intoxicated experimental animals (Abdel-Baset et al. 1997). This increase may be due to the abnormal dynamic properties of cellular membranes following exposure to hydrocarbon fractions present in kerosene and petrol fumes. Metabolism of aliphatic and aromatic hydrocarbons which are the major constituents of petroleum fumes as well as other xenobiotics have resulted in changes in the cell membrane due to reactive free radical species (Leighton et al. 1985; Bondy et al. 1995). The ratio of AST/ALT is also an important index for the measurement of toxicity. The decrease in the ratios in the animals exposed to kerosene and petrol fumes showed that the liver is likely to be most affected tissue.

Alkaline phosphatase activity in the animals exposed to kerosene and petrol fumes were significantly higher ($p < 0.05$) as compared to the control animals. This implies that damages may have occurred in the liver cells, since the activity of this enzyme in the serum is reported to be increased in liver damage (Abdel-Baset et al. 1997). Alkaline phosphatase is involved in the transport of metabolites across the cell membranes, protein synthesis, synthesis of certain enzymes, secretory activities and glycogen metabolism. The increase in this enzyme activity may not be unconnected with a disturbance in the transport of metabolites or alteration in the synthesis of certain enzymes as in other hepatotoxic conditions (Sharma et al. 1995).

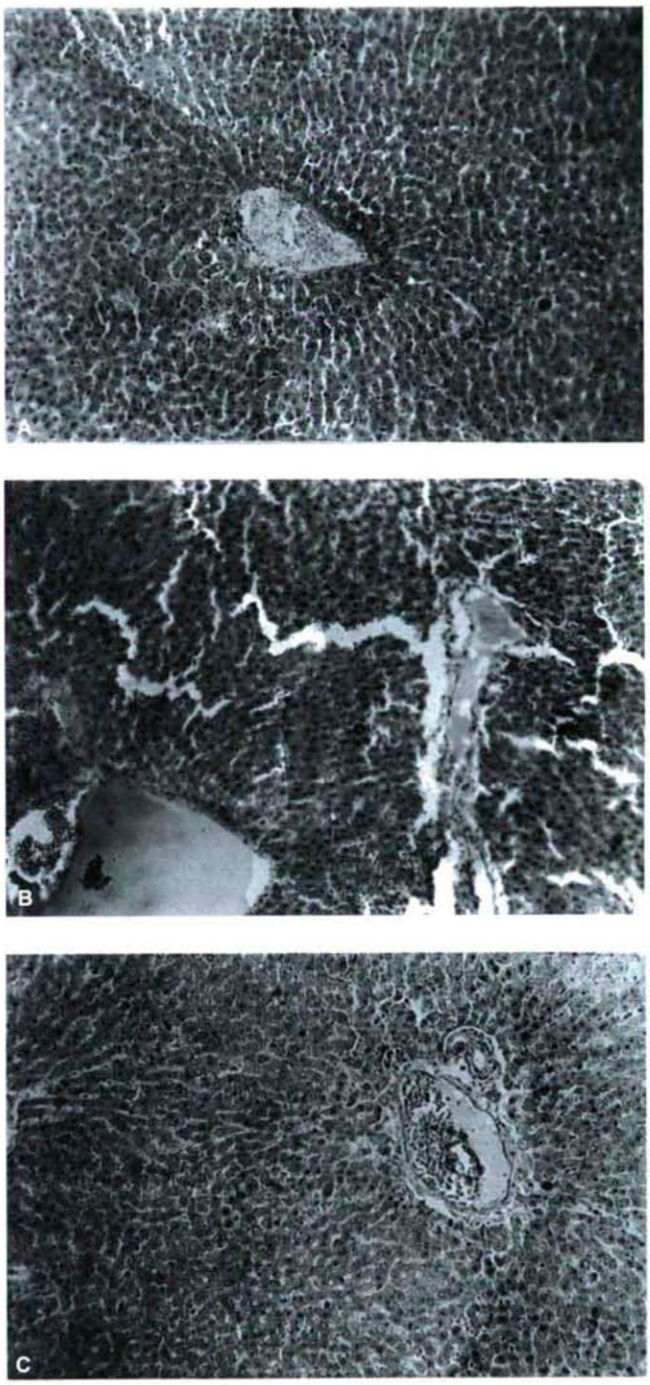

Figure 1. Photomicrograph of a typical liver section. (A) Control animal showing normal liver cell architecture. (B) Kerosene fumes-exposed animals show massive tissue degeneration. (C) Petrol fumes-exposed animals show liver necrosis. Magnification: 50x

The attendant effect of the exposure of experimental animals to kerosene and petrol fumes is that the reactive intermediates generated may have disrupted the cell membranes leading to enzyme leakage and tissue damage. Histological analysis of the liver tissues of the experimental animals (Fig. 1) indicates that frequent exposure to kerosene and petrol fumes affects the structural integrity of the liver cells. This implies that the liver is one of the major target organs of kerosene and petrol fumes-induced injury. The cumulative oxidative damage is therefore likely to be one of the underlying mechanisms responsible for the hepatotoxic effects of kerosene and petrol fumes, as observed in this study.

The significant increase ($p < 0.05$) in the levels of serum total cholesterol and triacylglycerol observed in this work is an indication that inhalation exposure to kerosene and petrol fumes also affect lipid metabolism. On one hand, lipid metabolism is affected once there is liver damage since the disturbance of cell membrane integrity is likely to cause some membrane lipids to be released into circulation, while on the other hand, it causes the tissue to compromise its effectiveness in regulating lipid metabolism. There is a likelihood that exposure to kerosene and petrol fumes predisposes the subject to atherosclerotic condition.

In conclusion, the results of this work suggest that repeated exposure to kerosene and petrol fumes may elicit hepatotoxicity, thereby impairing the normal liver function. Petroleum workers therefore should have regular medical check-up to ascertain their health condition. Further work is ongoing on the influence of kerosene and petrol fumes on oxidative metabolism.

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ARTICLE

Axial control of protein reserve mobilization during germination of indian bean (*Dolichos lablab* L.) seeds

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ABSTRACT The influence of embryonic axis and exogenously applied plant growth hormones on protein mobilization and development of proteases have been investigated in Indian bean (*Dolichos lablab* L. var lignosus) seeds during germination and post-germinative growth up to 10 days. Accumulation of free amino acids synchronized with rapid proteolysis and higher levels were maintained throughout the germination period. The presence of proteases (acid, neutral and alkaline) with three different pH optima increased in the early stages of germination and decreased later. The axis-excision affected the activities of proteases and protein degradation. Furthermore, the free amino acid content increased continuously in detached cotyledons throughout the germination period. Treatment with 1% casein hydrolysate to simulate the accumulation of free amino acids had a telling inhibitory effect on the proteases in attached and detached cotyledons. Exogenously applied phytohormones BA (Benzyl adenine), GA_3 (Gibberellic acid) or IAA (Indole acetic acid) resulted in stimulation of development of proteases as well as proteolysis in detached cotyledons. The two hypotheses, source-sink and hormonal stimulus both were influencing in the mobilization of food reserves and the growth of seedling. The results of the study supports the role of axis in protein mobilization regulating the development of proteases by providing phytohormone signals and regulation of their activity *in vivo* by a feedback mechanism.

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KEY WORDS

Dolichos lablab
proteolytic enzymes
embryonic axis
plant hormones
protein reserve mobilization
seed germination

The mobilization of storage proteins is one of the most important post-germinative event in the growth and development of seedling. During germination period, the storage proteins are degraded by a variety of proteases which convert the insoluble storage proteins into soluble peptides and free amino acids; these are mobilized to the embryonic axis to support its growth and also provide energy by oxidation of the carbon skeleton after deamination (Mayer and Poljakolf-Mayber 1982; Bewley and Black 1994; Shutov and Vaintraub 1987; Okamato and Minamikawa 1998; Muntz et al. 2001; Schlereth et al. 2001). The food reserve mobilization and its regulation in dicotyledonous seeds has been receiving attention and two hypotheses have been put forth to explain the role of axis in the process. First, the growing axis may act as *sink* to draw away the products of degradation, which may inhibit further development of enzymes and/or inhibit their activities. Second, the growing axis may produce the plant growth substance(s) that stimulate the synthesis of hydrolytic enzymes needed for food reserve mobilization in the cotyledons (Davies and Slack 1981; Bewley and Black 1994; Nandi et al. 1995). The mobilization of food reserves and the growth of seedling appear to be an efficiently synchronized process with embryonic axis influencing the two processes.

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In cereals, the proteases responsible for the degradation of storage proteins are synthesized in aleurone layer and the synthesis of the enzymes is upregulated by gibberellins and down regulated by ABA (Abscisic acid; Rogers et al. 1985; Ritchie et al. 2000). However, the role of the embryo or embryonic axis in the control of food mobilization in dicotyledonous seeds is less understood. There are only a few reports regarding the protein reserve mobilization, which is influenced by either source-sink process or hormonal stimulus and no reports are available for the operation of two processes. The mechanisms of axial control need to be examined thoroughly to arrive at a unified mechanism of axial control of reserve mobilization in dicotyledons and require extensive studies with different species. Hence, the present study is aimed at investigating the mobilization of storage proteins and the role of embryonic axis in the regulation of protein mobilization during germination of Indian bean (*Dolichos lablab* L. var lignosus) seeds. We reported in this study the role of axis in protein mobilization regulating the development of proteases by providing phytohormone signals (hormonal stimulus) and the regulation of their activity *in vivo* by feed backmechanism (source-sink).

Materials and Methods

Indian bean (*Dolichos lablab* L. *var lignosus*) seeds were

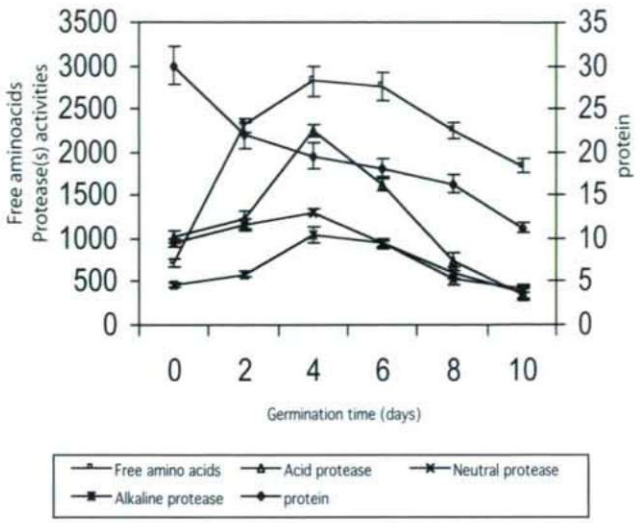

Figure 1. Changes in protein, free amino acid and the activities of acid, neutral and alkaline proteases in the cotyledons during the germination of Indian bean seeds.

procured from the Agricultural form of Andhra Pradesh Agricultural University, Rekulakunta, Anantapur, Andhra Pradesh, India. Healthy seeds of uniform size and weight were sorted and stored in a sterile plastic container until use. Seeds were surface sterilized with 0.1% $HgCl_2$ solution for 5 min and rinsed thoroughly with sterile distilled water. Soaking the seeds in distilled water for 12 h carried out imbibition. The water-imbibed seeds were germinated at room temperature for 10 days in sterile Petri dishes lined with moist filter paper. Sterile conditions were maintained by including 20 ppm of streptomycin sulphate in the incubation medium. Seedlings were withdrawn at designated time intervals and the cotyledons were carefully dissected out for analysis. The period of incubation (germination) was measured from the time when the imbibed seeds were transferred to the Petri dishes. Each experiment was carried out at least five times and each analysis was carried out in duplicate and averaged, unless otherwise stated.

To evaluate the influence of axis, the seed coat was removed after imbibitions and the cotyledons were separated from each other so that the axis remained attached to one of them. The cotyledons with (attached) or without (detached) axis were incubated with water or test solution under the same conditions.

Determination of free amino acids

An extract was prepared by boiling the cotyledons with 80% ethanol for 10 min. and centrifuged at 3,000 rpm for 10 min and the residue was re-extracted twice with hot alcohol and the pooled supernatants were filtered. The filtrate was suitably diluted and used for the estimation of amino acids by ninhydrin method (Raghuramulu et al. 2003).

Preparation of cotyledonary extract

The cotyledons were ground thoroughly in a pre-chilled mortar with chilled 0.05 M tris-HCl buffer, pH 7.2, containing 2 mM ß-mercaptoethanol. The extract was filtered and centrifuged at 10,000 rpm for 15 min. The supernatant was used for the estimation of proteins and assay of proteolytic enzymes. The results were expressed as µg/2 cotyledons.

Estimation of proteins

Protein content in the cotyledonary extract was estimated by the method of Lowry (1951). The results were expressed as mg/2 cotyledons.

Assay of proteolytic enzymes

Endopeptidase enzyme activity was measured by the modified method of Beevers (1968) using casein as substrate. The reaction mixture containing 1 ml of diluted enzyme extract, 1 ml of 1% casein (prepared in 0.1 N NaOH, pH adjusted to 7.0 with 0.1 N HCl), and 1 ml of appropriate buffer (0.1 M acetate buffer, pH 5.5, for acid protease, 0.1 M phosphate buffer, pH 7.3, for neutral protease and 0.025 M borate buffer, pH 8.8, for alkaline protease). Incubation was carried out for 1 h at 40°C. The reaction was arrested by the adding 1 ml of 20% trichloroacetic acid. The contents of the tube were kept at 4° C for 15 min and centrifuged at 3,000 rpm for 15 min. An aliquot of the supernatant was used for the determination of amino acids by ninhydrin method. The results were expressed as µmoles of amino acids released per hour in 2 cotyledons under experimental conditions.

Statistical analysis

Each value presented in figures and tables represent the arithmetic mean ± SE of five independent determinations, unless otherwise stated. The level of significance in between germination periods was calculated by DMR (Duncan Multiple Range) test.

Results

The maximal rates of protein depletion were observed during the first and last stages of germination (Fig. 1). Accumulation of free amino acids synchronized with rapid proteolysis and maximal rate of increase was observed in the first four days (250%, 30%) and then decreased marginally in between 2 to 15%. However, the level of free amino acids was still higher on day 10 by 2.5 fold, as compared to basal level on day 0. The cotyledonary extract of germinating Indian bean exhibited presence of caseinolytic activity at three different pH optima – 5.5, 7.3 and 8.8 (data not shown) – and the developmental profile of these three proteases (acid, neutral and alkaline) is depicted in Figure 1. The activities of all the three proteases increased uniformly up to day 4 and then

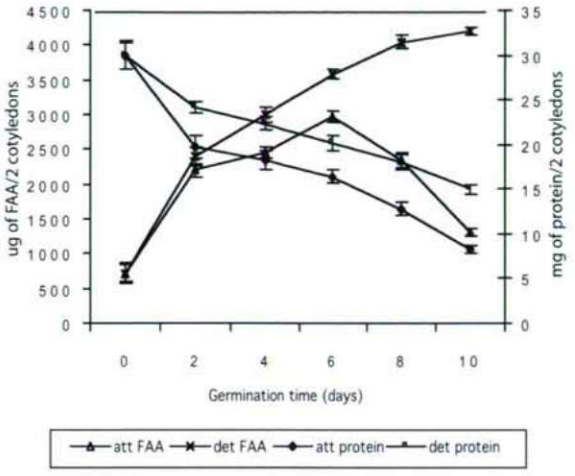

Figure 2A. Changes in protein and free amino acids in attached and detached cotyledons of Indian bean during germination.

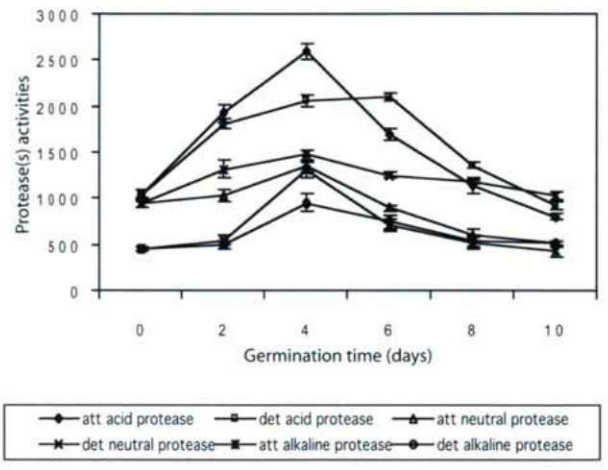

Figure 2B. Changes in proteases (acid, neutral and alkaline) in attached and detached cotyledons of Indian bean during germination.

gradually fell. However, the activity of acid proteases was higher throughout the germination period compared with the activities of neutral and alkaline proteases.

Role of axis on protein degradation

The influence of axis on protein degradation and proteases development was evaluated by using attached and detached cotyledons. Concomitant with the fall in the protein content, the free amino acid level in attached cotyledons increased maximally (3 fold) by day 2 and continued to increase up to day 6 and then declined further (Figure 2A). By contrast, a slower rate of degradation of protein was observed in detached cotyledons with maximal rates (16-20%) between day 0 and 2. The overall loss of protein in detached cotyledons was only 45% of the basal level while in attached cotyledons it was 72%. The free amino acid level in detached cotyledons, unlike in attached cotyledons, continued to increase throughout the germination period. It is pertinent to note that in the absence of embryonic axis the protein degradation retarded with accumulation of free amino acids.

The gradual development of all the three proteases in attached cotyledons increased up to day 4 and declined thereafter (Fig 2B). However, the developmental activities of all proteases in detached cotyledons were slow and delayed and maximal activity of acid protease observed on day 6 was nearly 20% lesser than the maximal activity observed on day 4 in attached cotyledons.

End product regulation of protein degradation

The possibility of the accumulation of free amino acids, end products of proteolysis, might bring about a repression of enzyme synthesis and/or inhibit the activities of proteases by feedback mechanism was examined. Germination in the presence of casein hydrolysate retarded the proteolytic process in attached and detached cotyledons compared to the cotyledons incubated in the absence of casein hydrolysate (Table 1). However, the accumulation of free amino acids was higher and the protein degradation was lower in detached cotyledons. The developments of all the three proteases were greatly affected and the activities on day 10 were one-third to one-fourth of the basal level in the presence of caseinhydrolysate.

Effect of exogenously applied plant growth hormones on protein degradation

Exogenously applied plant hormones (BA, GA_3 or IAA) had profound effect on protein degradation in detached cotyledons. Maximum stimulatory effect on the protease(s) activities in detached cotyledons was observed at concentrations of 0.01 mM of BA, GA_3 or IAA (Fig. 3), although perceptible changes were observed between 0.001 to 0.1 mM. All the hormones at these concentrations enhanced protein degradation as reflected by decrease in protein and increase in free amino acid level in detached cotyledons. It is pertinent to note that exogenously applied hormones did stimulate the development of proteases and proteolytic processes in detached cotyledons and this stimulation was even higher than that was observed in attached cotyledons.

Discussion

Solvation of insoluble proteins, activation of pre-existing enzymes and/or *de novo* synthesis of enzymes, and degradation of storage proteins are apparently a chain of events leading to the transport of products to the growing axis for the

Table 1. Effect of 1% casein hydrolysate on protein mobilization in attached and detached cotyledons during germination. The attached and detached cotyledons were incubated in water or 1% casein hydrolysate solution for the time intervals indicated and analyzed for protein, FAA (Free amino acids) and protease activities. Each value is mean ±SE of five values.

Germination Time (Days)	Protein (mg/2 cotyledons)		FAA (mg/2 cotyledons)		Protease(s) activity (μ moles of amino acids released /h/2 cotyledons)					
					Acid		Neutral		Alkaline	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Attached cotyledons										
0	30.00±0.62	30.00±0.62	0.72±0.05	0.72±0.05	1022±63	1022±63	950±43	950±43	455±20	456±22
2	19.72±0.64	28.02±0.59	2.20±0.06	6.73±0.5	1928±20	1052±38	1088±58[a]	970±55[a]	548±43[b]	576±44[b]
4	18.30±0.63	24.78±0.40	2.46±0.10	6.63±0.4	2592±92	1084±96	1344±40	1032±47	1308±88	434±60
6	16.40±0.78	22.69±0.42	2.98±0.04	5.00±0.5	1699±66	704±93	863±32	704±36	640±64	394±38
8	12.80±0.45	19.24±0.36	2.34±0.06	3.53±0.3	1129±59	674±92	584±43	464±52	456±58	242±25
10	8.26±0.12	16.80±0.61	1.24±0.04	2.53±0.2	790±30	372±30	494±14	264±25v	413±13	164±18
Detached cotyledons										
0	30.00±0.62	30.00±0.62	0.72±0.05	0.72±0.05	1022±63	1022±63	950±43	950±43	455±20	456±22
2	24.12±0.54	30.10±0.9	2.38±0.05	6.19±0.34	1803±64	834±48	1314±43	716±40	488±35[c]	466±26[c]
4	22.28±0.70	27.50±0.5	2.96±0.09	6.83±0.74	2086±132	648±54	1474±42	796±92	956±70	480±51
6	20.08±0.31	25.80±0.5	3.57±0.05	7.12±0.56	2292±74	496±33	1244±63	536±45	743±13	460±59
8	17.98±0.80	24.92±0.5	4.03±0.04	8.51±0.50	1362±84	280±25	1180±23	440±23	535±23	392±37
10	14.98±0.52	23.15±0.9	4.10±0.03	9.53±0.60	894±64	196±27	1028±25	286±25	505±14	322±22

Mean ± SE followed by the same letter do not differ according to DMR test at 5% level of significance ($P < 0.01$).

synthesis of new proteins and other nitrogenous compounds (Bewley and Black 1994; Callis 1995; Shewry et al. 1995). Consistent with these general phenomena, we (Ramakrishna and Ramakrishna Rao 2005) reported previously that the total protein of Indian bean is depleted during germination period. The decrease in protein content in the cotyledons during germination is also reported in lima bean (Heywood and Gainer 1974), horse gram (Karunagaran and Ramakrishna Rao 1990; Rajeswari and Ramakrishna Rao 2002), lupine (Nandi et al. 1995), *Vigna mungo* (Taneyama et al. 1996) and *Vicia sativa* (Misra and Kar 1990). However, in the cotyledons of vetch during germination the amounts of the amino acids and proteins did not change (Schlereth et al. 2001). The existence and development of acid, neutral and alkaline proteases have also been noted in the cotyledons of germinating legume and non-legume seeds (Misra and Kar 1990; Shastry and John 1991). Increase in proteolytic activity with concomitant reserve protein depletion agrees with the findings of earlier works in other legume seeds: *Phaseolus vulgaris* (Senyuk et al. 1998), *Vigna mungo* (Taneyama et al. 1996), *Lupinus albus* (Ferreira et al. 1995), *Vicia sativa* (Schlereth et al. 2001), and horse gram (Karunagaran and Ramakrishna Rao 1990; Rajeswari and Ramakrishna Rao 2002).

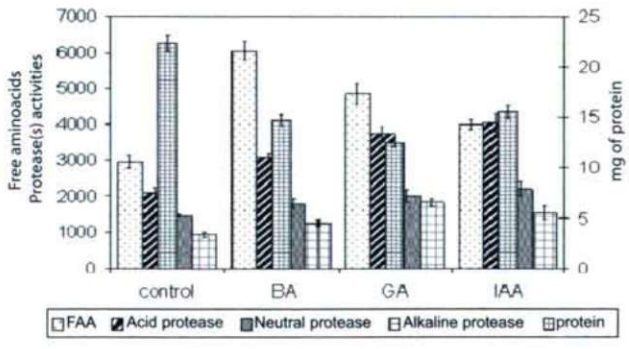

Figure 3. Changes in the levels of protein, free amino acids and activities of proteases (acid, neutral and alkaline) in detached cotyledons incubated in the presence of hormone (0.01 mM) for 4 days compared with corresponding controls incubated in water.

The regulation of proteolysis in the Indian bean cotyledons by feedback inhibition by the end products is favored by several observations made in this study: (1) the amino acid content in detached cotyledons increases continuously during the time course of study and the depletion of protein and the development of proteases are retarded compared with attached cotyledons (Figs. 2A, 2B); (2) the activities of proteases are also retarded when the cotyledons (attached and detached) are incubated with casein hydrolysate substantiating the inverse relationship noticeable between the amino acid content and the activities of proteases in detached cotyledons (Table 1). In agreement with these results, the protein mobilization in buckwheat is subject to feedback inhibition by accumulation of protein degradation products *in vitro* and *in vivo* (Dunavsky and Belozersky 1993). Treatment with casaminoacids inhibited the growth of mung bean seedlings with a parallel inhibition of the development of vacillin peptidohydrolase activity in the cotyledons (Kern and Chrispeels

1978). The findings of the present study support the notion that the feedback regulation is highly likely to be involved in the control of protease development during the germination of Indian bean.

Exogenously applied plant growth hormones (BA, GA_3 or IAA) in the present study stimulated the development of proteases and proteolysis in detached cotyledons indicating the possibility of involvement of hormones in the axial control of development of proteases. In line with present data, Taneyama et al. (1996) showed that the levels of endopeptidase activity was doubled when GA_3 (10-100 μM) was applied to the detached cotyledons of *Vigna mungo* seeds and they suggested the possibility that gibberellins (GA_3) synthesized in axis of seedlings are transported to cotyledons and that the biologically active from GA_3 triggers the expressions of SH-EP in cotyledons. Similarly in lupine (Nandi et al. 1995) and squash cotyledons (Ashton 1976) the plant hormone, cytokinin could substitute for the embryonic axis with respect to endopeptidase development. The effect of the removal of the axis on the protease level might be ascribable to the removal of endogenous source of plant hormones that might stimulate the synthesis of the enzyme in the cotyledons.

In conclusion, the results obtained from this study indicate that the embryonic axis is required for reserve protein mobilization in the cotyledons of germinating Indian bean. The two hypotheses, source-sink and hormonal stimulus were influencing in the mobilization of food reserves and the growth of seedling. While the phytohormone signals transmitted from the axis to the storage tissue appear to be necessary for the development of proteases and regulated their activity *in vivo via* feedback mechanism by the levels of end products at the site of reserve food breakdown. The hormonal effect on protease may be due to the induction of enzyme synthesis or suppression of the degradation of enzyme or both. Further investigation into these possibilities is in progress to identify whether the hormonal effect is through induction at early stages of germination or due to slower degradation of protease in the later stages of germination.

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ARTICLE

Quantitative flavonoid variations of *Artemisia vulgaris* L. and *Veronica chamaedrys* L. in relation to altitude and polluted environment

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ABSTRACT Influence of altitudinal gradient and polluted environment on externally accumulated flavonoid aglycones were examined. Contents of apigenin in *Veronica chamaedrys* L. and quercetin 3,7,3'-trimethyl ether in *Artemisia vulgaris* L. were determinated. The highest apigenin level was found in populations at alpine regions, whereas no relation was found between quercetin 3,7,3'-trimethyl ether and altitude. The largest amounts of quercetin 3,7,3'-trimethyl ether was found in samples collected from industrial polluted habitats. Ecological significance on external flavonoid aglycones is discussed. **Acta Biol Szeged 49(3-4):29-32 (2005)**

KEY WORDS

flavonoid aglycones
altitudinal gradient
pollution
Artemisia vulgaris
Veronica chamaedrys

Flavonoid variations are considered as a phytochemical adaptation to the abiotic and biotic environment by Dixon and Pavia (1995). Lipophilic flavonoid aglycones are accumulated on the plant surface (Wollenweber 1990). It has been assumed that this surface disposition was determined by the chemo-ecological functions of aglycones. There are extensively data that flavonoid synthesis is influenced by different abiotic and biotic factors: UV light radiation, drought, ozone, phytopathogens and insect-deterrent (Tomas-Barberan et al. 1988; Midiwo et al. 1990; Cuadra et al. 1997; Cooper-Driver and Bhattacharya 1998; Lalova 1998; Markham et al. 1998; Simmonds 1998; Chaves et al. 2001; Saleem et al. 2001).

Artemisia vulgaris L. (Asteraceae) and *Veronica chamaedrys* L. (Scrophulariaceae) are perennial herbs, widely distributed in different habitats, from 0 to 1800 meter above sea level (m asl) for *A. vulgaris* populations and from 0 to 2300 m asl for *V. chamaedrys* populations. Simple flavonol methyl ethers have been reported for West-European populations of *A. vulgaris* (Valant-Vetschera et al. 2003) and flavone derivatives of *V. chamaedrys* populations (Nikolova et al. 2003).

Although the intraspecific flavonoid variation has been documented in numerous studies (cited by Bohn1987), only a limited number of reports treated the subject of flavonoid changes along altitudinal gradient (McDougal and Parks 1984) and environmental pollution (Loponen et al. 1998). In the present study we examine external flavonoid aglycones variations of *Veronica chamaedrys* and *Artemisia vulgaris* across altitudinal gradient and polluted environment.

The sampling of populations from habitats with different conditions allowed us to assess the intraspecific variations and main ecological trends of flavonoid accumulation.

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Materials and Methods

Plant material

The plant samples were formed from aerial parts of blossoming plants collected from natural habitats on 24 populations of *A. vulgaris* during 1999, and on 12 populations of *V. chamaedrys* during 2001 in Bulgaria. The sampling sites were chosen to cover regions with different type and degree of environmental pollution as well as at different altitude. Altitudinal gradient includes populations of *V. chamaedrys* and *A. vulgaris* situated at various altitudes from 700 to 2290 m asl at Vitosha mountain (Bulgaria). Pollution gradient includes populations of *A. vulgaris* from habitats with industrial, traffic and background pollution. We examined influence on environmental pollution only on *A. vulgaris* populations because *V. chamaedrys* populations have a rare occurrence in polluted regions. All habitats are situated on similar altitude in the same geographical region. Industrial contamination is mostly by lead (Pb) and iron (Fe) ions and not so much by cooper (Cu) and zinc (Zn). Main toxic emissions are aromatic aerosols, ozone (O_3), sulphur oxide (SOx), hydrogen sulphide (H_2S), carbon oxide (CO), dust (Topalov 2001). Voucher specimens were deposited at the Herbarium in the Institute of Botany, Sofia, Bulgaria.

Sample preparation

Plant exudates were prepared from air-dried, not grounded aerial parts (5 g) by rinsed with acetone 2x20 ml for 5 min to dissolve the material accumulated on leaf and stem surfaces. After evaporation of acetone, the dried extracts were dissolved in methanol for further TLC analysis.

Table 1. Voucher numbers (SOM) and apigenin content in the samples of *V. chamaedrys* along altitudinal gradient.

SOM	Altitude [m asl]	Apigenin [µg/]±SD*
Co476	750	0.57±0.06
Co669	800	0.80±0.08
Co615	1000	1.60±0.08
Co618	1200	0.42±0.07
Co619	1250	0.47±0.06
Co623	1340	1.00±0.08
Co672	1600	1.56±0.06
Co621	1810	2.08±0.07
Co617	1870	1.80±0.06
Co616	1950	2.12±0.06
Co675	2200	1.61±0.07
Co614	2290	2.05±0.05

*Results are average values of three measurements; m asl: meters above sea level.

Table 2. Voucher numbers (SOM) and quercetin 3,7,3'-trimethyl ether content in the samples of *A. vulgaris* along altitudinal gradient.

SOM	Altitude [m asl]	Quercetin 3,7,3'-trimethyl ether [µg/g]±SD*
Co515	750	0.28±0.06
Co526	800	0.35±0.03
Co517	900	0.34±0.09
Co577	1000	0.32±0.03
Co688	1200	0.29±0.04
Co560	1300	0.35±0.04
Co518	1400	0.32±0.04
Co556	1440	0.29±0.05
Co513	1550	0.30±0.03
Co512	1800	0.28±0.06

*Results are average values of three measurements; m asl: meters above sea level.

Flavonoid quantification

Toluene-dioxan-acetic acid (95:25:4) mixtures were used for the development of plates. Migration distance was 90 mm. 60 µl of *V. chamaedrys* exudates and 40 µl of *A. vulgaris* exudates with unknown concentrations were spotted and developed on Merck aluminum sheets Kiselgel 60 F_{254} (0.2 mm thin layer, 10 x 20 cm) together with standards. Compounds were visualized after spraying with "Naturstoffreagenz A" reagent. The fluorescence emissions of apigenin (Rf = 0.34) and quercetin 3,7,3'-trimethyl ether (Rf = 0.60) were snapped under UV radiation=336 nm with a digital camera, and the images were analyzed by QuantiScan 2.1® Biosoft software (Nikolova et al. 2004). The apigenin and quercetin 3,7,3'-trimethyl ether contents of the exudates were calculated from the densitogram peak areas by comparing to three standards (0.75, 1.5, 3 µg/spot of apigenin and 0.75, 1, 1.5 µg/spot of quercetin 3,7,3'-trimethyl ether) placed on the same plate.

Flavonoid aglycones used as reference compounds in the TLC analysis, namely quercetin 3,7,3'-trimethyl ether and apigenin have been isolated and identified respectively from *A. vulgaris* and *V. chamaedrys* in a previous study (Nikolova 2002).

Table 3. Voucher numbers (SOM) and quercetin 3,7,3'-trimethyl ether content of in the samples of *A. vulgaris* from habitats with different type of pollution.

SOM	Habitat information	Quercetin 3,7,3'-trimethyl ether [µg/g] SD*
industrial polluted zone		
155134	Metal works "Kremikovstsi", 800 m asl	0.48±0.03
Co 573	Chemical works "Verila", 700 m asl	0.46±0.03
Co 510	Railway station "Yana" between metals works and uranium mine, 600 m asl	0.63±0.08
Co663	v. "Ravno pole", there is flying toxic fragments to air, 500 m asl	0.47±0.09
Co511	Uranium mine "Buhovo", 900 m asl	0.52±0.05
traffic polluted zone		
Co662	Sofia, boulevard, 550 m asl	0.40±0.03
Co566	Sofia, main street, 500 m asl	0.35±0.06
Co647	Sofia, highway 550 m asl	0.44±0.03
Co694	Sofia, metro station 500 m asl	0.40±0.08
Co571	Sofia, airport 550 m asl	0.36±0.03
background polluted zone		
Co683	Ljulun mountain, 700 m asl	0.28±0.01
Co515	Vitosha mountain, 700 m asl	0.28±0.06
Co558	Vitosha mountain, 800 m asl	0.22±0.02
Co574	Vitosha mountain, 900 m asl	0.34±0.09
Co606	Lozen mountain, 800 m asl	0.32±0.02

*Results are average values of three measurements; m asl: meters above sea level.

Results and Discussion

Flavonoid aglycones variation along altitudinal gradient

A TLC survey of apigenin variations over altitudinal interval 700-2300 m asl on 12 populations of *V. chamaedrys* was performed and the results are given in Table 1. The apigenin content ranged from 0.47 to 2.17 µg/g of the dried leaf weight. The largest amounts of apigenin was found in the samples at the alpine regions. This pattern could be explained with the xeric alpine habitats and presumable UV-screen function of the leaf surface flavonoids. There are many reports demonstrating that flavonoid synthesis is induced by UV radiation (Lois 1994; Cuadra et al. 1997; Lalova 1998; Markham et al. 1998; Hofmann et al. 2000). Our results are in agreement with previous studies, which report a higher accumulation of exudate flavonoids as a response to more xeric habitats (Wollenweber 1990; Chaves at al. 1997; Willliams et al. 1997; Valant-Vetschera and Wollenweber 2001).

The quantitative analysis on flavonoid content in the 10 populations of *A. vulgaris* over altidutinal inerval 800-1800 m asl showed that the altitude does not have a significant effect on quercetin 3,7,3'-trimethyl ether synthesis (Table 2). We suppose that structural differences on apigenin and quercetin 3,7,3'-trimethyl ether are the reason for their different metabolisms along altitudinal gradient. The O-methylation of the hydroxyl substations inactivates anthioxidant activities of the flavonoids (Cao et al. 1997; Burda and Oleszek 2001). In this way apigenin was more efficient antioxidant than quercetin 3,7,3'-trimethyl ether. The antioxidant activity on aglycones is important for their protective role because UV radiation induces production of free radicals (Foyer et al. 1994).

Flavonoid aglycones variation in dependence of type on environmental pollution

The quantification of quercetin 3,7,3'-trimethyl showed considerable differences among the populations of *A. vulgaris* from the habitats with different type of pollution (Table 3). The populations from the industrial polluted habitats displayed high content on quercetin 3,7,3'-trimethyl ether. Increases of phenolic compounds and flavonoids as a result of pollution impact have been observed in tree species (Loponen et al. 1997, 1998; Giertych et al. 1999), but the reasons for this increase are unclear. Chaves et. al. (1997) concluded that accumulation of methylated flavonoids prevent water loss and increase the general stress tolerance of the plants.

The present study reported infraspecific flavonoid variations of *A. vulgaris* and *V. chamaedrys* in relation to altitudinal gradient and polluted environment. The apigenin accumulation in *V. chamaedrys* was increased at the alpine regions, while the content of quercetin 3,7,3'-trimethyl ether in *A. vulgaris* appeared to be independent of altitude. The synthesis of quercetin 3,7,3'-trimethyl ether was influenced positively by environmental pollution. The data support the postulated ecological significance on external flavonoid aglycones.

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ARTICLE

Phytohormones dynamics during flowering initiation in carrots

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ABSTRACT This study was aimed on investigation of phytohormones dynamics in carrot (*Daucus carota* L.) during different flowering initiation stages. Different levels of development were needed for photo- and thermo-induction; tipically 5 and 9 leaves in rosette for the first and second flowering induction stages, respectively. The process of carrot flowering initiation and morphogenesis was studied in a phytotron facility. High-performance liquid chromatography was used for separation of phytohormones. The best rate of development was found during exposure to florally inductive effect with low temperature either a short day or long day photoperiod in carrots with 9 leaves in rosette. Temperature influence had higher effect on phytohormones biosynthesis than differences in photoperiod. Antagonistic as well as stimulatory steps were involved during hormonal action and the balance of these determined the final effect of even a single hormone. The ratio of investigated phytohormones had substantial influence on flowering initiation processes. An increase of gibberellic acid content in evocation stage II and decrease in flower initiation stage determined faster stem elongation and bud formation. Decrease of abscisic acid and increase of gibberellic acid level in evocation stage II could be related with fast flowering induction processes. The highest indol-3 acetic acid concentration in evocation stage II induced the formation of inflorescence axis structures.
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KEY WORDS

carrot
evocation
flowering initiation
phytohormones

There exist several hypotheses, concepts and theories for explaining the mechanism of plant transition to the generative development (Chailakhyan 1988; Jordan 1993). In most of these theories, various aspects of the hormonal regulation of flowering were analyzed. It has been proposed that phytohormones are involved in the metabolism pathway, which occurs after light perception and transduction of the initial signal into a physiological effect (Kraepiel and Miginiac 1997). The carrot plant flowers only after vernalization, *i.e.*, low temperatures are needed for flower initiation (Dias-Tagliacozzo and Válio 1994). Reproductive success of plant largely depends on the correct timing of floral induction. This is the reason why the initiation of flowering is highly regulated by environmental cues exhibiting regular seasonal changes, such as photoperiod and temperature, and by the developmental stage of the plants (Bernier et al. 1993). The end of the juvenile period is linked to plant's utter preparation for photo- and thermo induction processes. In carrot ontogenesis this moment corresponds with the beginning of carrot root thickening. At that moment plants form 8 assimilating leaves. Photo- and thermo induction mechanisms are not interrelated, they can proceed at different time and stipulate different effects (Duchovskis et al. 2003). According to Duchovskis (2004), there are two periods in flowering induction and evocation. The first period of flowering induction is photoinduction (5 leaves in rosette for carrot). Metabolites of photomorphogenetic system are transported to apical meristems, where they can de-block the genes of inflorescence axis formation. From this moment evocation period first starts which ends with the formation of the inflorescence axis. The second period of flowering induction of wintering plants is thermo induction (vernalization; 9 leaves in rosette for carrot); its metabolites determine the formation of inflorescence axis structures (evocation period second). These processes requite in flower initiation and differentiation (Duchovskis 2004).

The aim of this study was to investigate the dynamics of phytohormones in carrot during different flowering initiation stages.

Materials and Methods

Carrot *Daucus carota* L. var. *Garduolė 2* was initially grown in vegetative tumbler 54x34x15 cm size placed in a greenhouse (16 h photoperiod and 21/16°C day/night temperature). Peat (pH ~6) was used as the substrate. Carrots with 5 and 9 leaves in rosette, respectively, were removed from the greenhouse and placed in phytotron chambers with different conditions for 120 days: EXP1 – photoperiod of 0 h and 4°C temperature, EXP2 – photoperiod of 8 h and 4°C temperature, EXP3 – photoperiod of 16 h and 4°C temperature,

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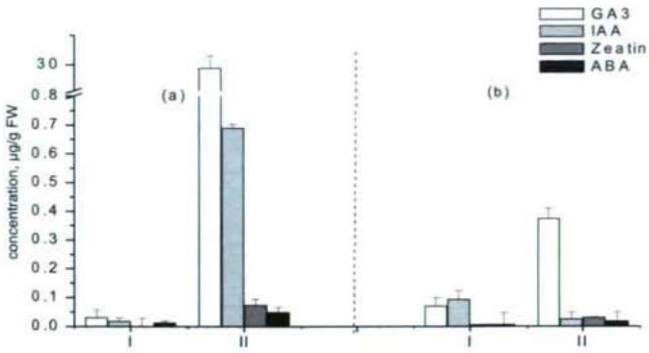

Figure 1. Changes in phytohormone concentrations in carrot rosette before (I) and after (II) flowering induction. (a) 5 leaves in rosette. (b) 9 leaves in rosette.

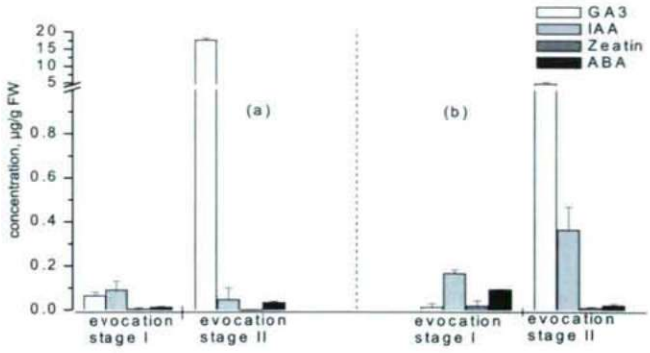

Figure 2. Changes in phytohormone concentrations in carrot during various flowering initiation stages. Photoperiod of 0 h and 4°C temperature (EXP1). (a) 9 leaves in rosette. (b) 5 leaves in rosette.

EXP4 – photoperiod of 8 h and 21/16°C temperature, EXP5 – photoperiod of 16 h and 21/16°C (day/night) temperature. After that organogenesis processes (Kuperman et al. 1982) were investigated with the photoperiod of 16 h and 21/16±2°C (day/night) temperature maintained. Plants were investigated under illumination using HPI-T lamps (Philips).

Samples for high-pressure liquid chromatography (HPLC) were prepared by grounding 1 to 2 g of fresh tissue per sample into powder under liquid nitrogen treatment. The samples were pre-purified using solid-phase extraction with NH_2-cartridge columns. The prepared samples were stored in vials at 4°C, as proposed by Wang et al. (2003).

Analysis of gibberellic acid (GA_3), indolyl-3-acetic acid (IAA), abscisic acid (ABA) and zeatin was performed using a Shimadzu HPLC model 10A chromatographer equipped with DAD detector (SPD-M 10A VP), column oven (CTO-10AS VP), degasser (DGU-14A), and two pumps (LC-10AT VP) enabling use of concentration gradient of the mobile phase. Separation and detection were performed on an Inertsil ODS-2 column (150 x 4.6 mm^2). Mobile phase gradients of 40% methanol containing 1% acetic acid for GA_3, 45% methanol in 1% acetic acid for IAA, 50% methanol in 1% acetic acid for zeatin, and 55% methanol in 1% acetic acid for ABA were used. The wavelengths of 254 nm, 280 nm, 270 nm, and 254 nm were set in the DAD detector for GA_3, IAA, zeatin, and ABA, respectively. The total run time for the separations at a flow rate of 1 mL/min was approximately 10 min.

The following chemicals were used: isopropanol (POCH, Poland), imidazole, GA_3, IAA, ABA and zeatin (Sigma-Aldrich, Germany), NH_2 -columns (Supelco, USA), methanol and hexane (LaChema, Czech Republic), acetic acid (BOH, England).

Results

The content of GA_3 and IAA dramatically increased after flowering induction, especially for carrots with 5 leaves in rosette (Fig. 1).

Under treatment with photoperiod of 0 h and 4°C temperature, the development of carrots stopped after evocation stage II. The concentration of GA_3 increased after evocation stage I in both cases, but the higher concentration was in the apex zone in carrots with 5 leaves in rosette (Fig. 2). As the concentration of GA_3 was low, the small increase of ABA was observed in evocation stage I (Fig. 2b). The content of IAA was higher in carrot with 9 than with 5 leaves in rosette.

Elements of inflorescence axis were formed (organogenesis stage IV) and further flowering initiation stages were reached faster by carrots with 9 leaves in rosette. However, the development of carrots with 9 leaves in rosette in different treatments was not identical. The growth of generative organs was the best in treatments EXP2 and EXP3, but the development of carrots with 9 leaves in rosette was faster than with 5 leaves in rosette (Table 1). The general tendency of GA_3 biosynthesis is observed in all treatments: low GA_3 levels were in evocation stage I, and the GA_3 content decreased during flower initiation (except of EXP2, carrots with 9 leaves in rosette). During flowering initiation stages the concentration of GA_3 was higher under treatment with low positive temperatures (EXP2 and EXP3) than under treatment with high temperatures (EXP4 andEXP5). Extremely high IAA concentrations were detected under treatment with high temperatures during evocation stage II in carrot with 5 leaves in rosette both in a short day (SD) or long day (LD) photoperiod (EXP4 and EXP5). The increase of IAA amount during evocation stage II and decrease during flower initiation was observed in all treatments in carrots with 5 leaves in rosette. However, the increase of IAA biosynthesis during all flowering initiation stages was observed for carrots with 9 leaves in rosette, except under treatment with low positive temperatures and LD photoperiod (EXP3). The highest ABA content was under treatment with a short day photoperiod and low positive temperatures (EXP2) in carrots with 9 leaves in rosette during evocation stage I and dramatically decreased in evocation stage II (Fig. 3).

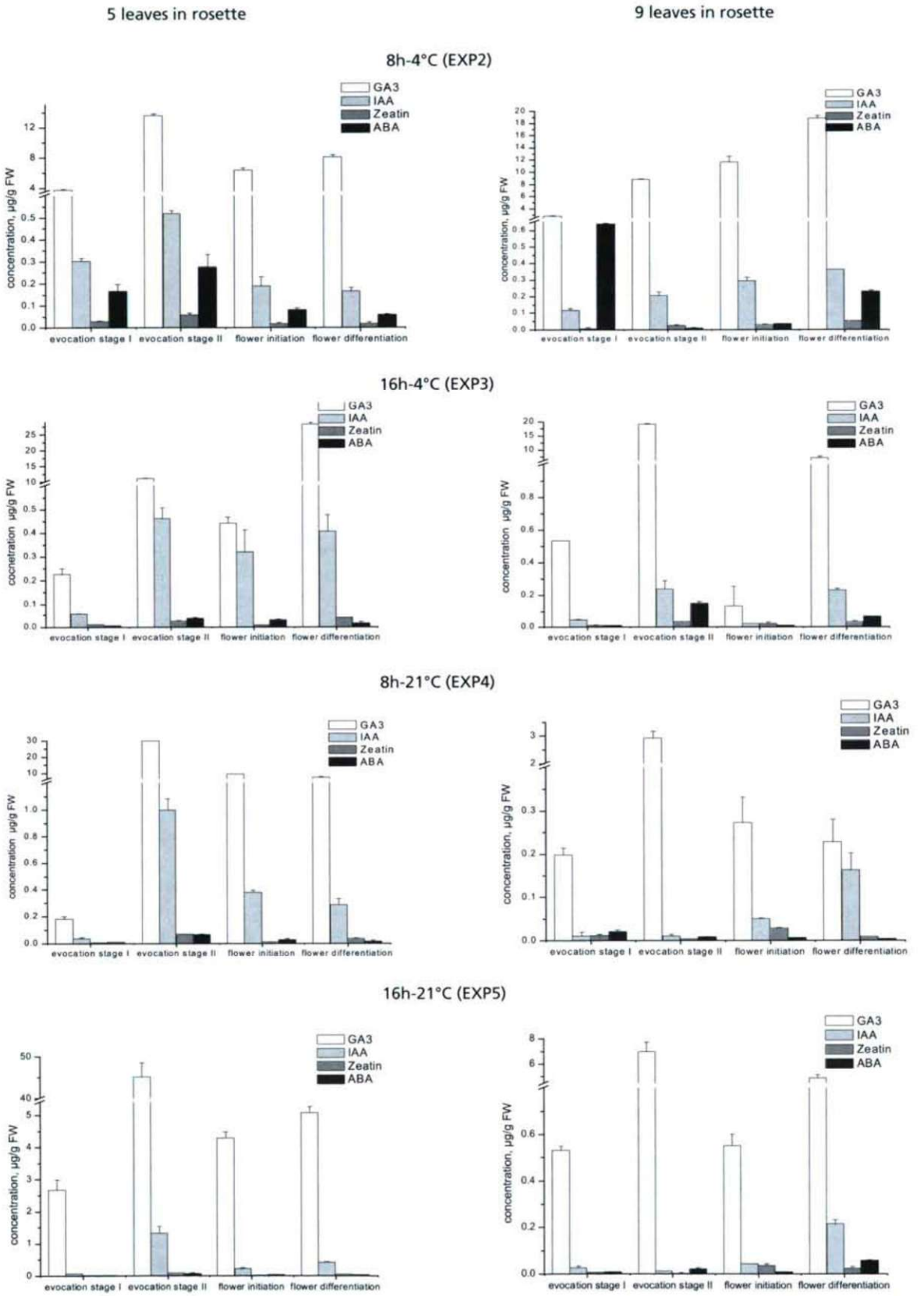

Figure 3. Changes in phytohormone concentrations in carrot under treatments with different photo and thermo periods.

Table 1. The intensity level of carrot development processes during different flowering initiation stages.

Treatment	Flowering initiation stage: Evocation stage I (organogenesis stage III) I	Evocation stage I (organogenesis stage III) II	Evocation stage II (organogenesis stage IV) I	Evocation stage II (organogenesis stage IV) II	Flower initiation (organogenesis stage V[a]) I	Flower initiation (organogenesis stage V[a]) II	Flower differentiation (organogenesis stage V[b], V[c]) I	Flower differentiation (organogenesis stage V[b], V[c]) II
EXP1	+	+	+	+	-	-	-	-
EXP2	++++	+++++	++++	+++++	++++	+++++	++++	+++++
EXP3	++++	+++++	++++	+++++	++++	+++++	++++	+++++
EXP4	++	+++	++	+++	++	+++	++	+++
EXP5	++	+++	++	+++	++	+++	++	+++

I - 5 leaves in rosette; II - 9 leaves in rosette.
"+/-" - development intensity level in carrot.

Discussion

As it is known, different development levels are needed for photo and thermo induction in carrots (Duchovskis et al. 2003; Duchovskis 2004). Photo- and thermo induction mechanisms are not interrelated. For the first stage of flowering induction (photo induction) 5 leaves in rosette are needed. From this moment starts the first evocation stage which ends with the formation of the inflorescence axis. After that the second evocation stage (thermo induction) starts. During these processes the formation of inflorescence axis elements is complete. At least 9 assimilating leaves are needed for the reaction to thermo induction (Duchovskis et al. 2003). The development of the investigated plants was unequal. Environmental cues are perceived by different organs in the plant and promote endogenous stimuli that signal the apical meristem (Lejeune et al. 1991). Biennial plants cannot be vernalized as imbibed seeds or young seedlings but rather must reach a critical age or developmental stage before vernalization can occur (Lang 1986). The shoot apex must be exposed to cold for vernalization to occur. This is consistent with vernalization causing the apical meristem to acquire competence to flower. In many species the plant hormone gibberellin plays a role in the regulation of flowering (Michaels and Amasino 2000). Could an increase in endogenous GA amount or an alteration of GA metabolism be a mechanism for the promotion of flowering without inductive photoperiods or cold treatment? Our results showed that an increase of GA_3 content in evocation stage II and decrease in flower initiation stage determines faster stem elongation and bud formation. Carrots under treatment with low temperatures showed the more rapid development. There is an assumption that changes in GA_3 quantity could be related to cold treatment, while changes in photoperiod did not give such results. It is known that both antagonistic as well as stimulatory steps are involved during hormonal action and the balance of these determines the final effect of even a single hormone (Johri and Mitra 2001). In several responses, such as flowering initiation, GA and ABA act antagonistically. Under treatment with SD photoperiod and low temperatures in evocation stage I the high ABA and low GA_3 concentrations were detected. After that decrease of ABA and increase of GA_3 level in evocation stage II can be related with fast flowering induction processes. Auxins are typically associated with cell elongation, while auxin and cytokinin act synergistically to regulate the process of cell division (Johri and Mitra 2001; Merkys et al. 2003). According to Duchovskis (2003, 2004), evocation period first ends with the formation of the inflorescence axis. Metabolites of the second period of flowering induction determine the formation of inflorescence axis structures (evocation period second). The common tendency of IAA biosynthesis was observed in all treatments for carrots with 5 leaves in rosette. The highest IAA concentration in evocation stage II may induce the formation of inflorescence axis structures. It seems that temperature influence has higher effect on IAA biosynthesis than differences in photoperiod.

Conclusions

1. The best rate of development was during exposure to florally inductive effect with low temperature either a short day or long day photoperiod in carrots with 9 leaves in rosette, when plants has a competency to accept influence of thermo induction.
2. Temperature influence has higher effect on phytohormones biosynthesis than differences in photoperiod.
3. It seems presumptively that the ratio of investigated phytohormones has substantial influence on flowering initiation processes in carrots:
- an increase of GA_3 content in evocation stage II and decrease in flower initiation stage determines faster stem elongation and bud formation;
- a decrease of ABA and increase of GA_3 level in evocation stage II can be related with fast flowering induction processes;
- the highest IAA concentration in evocation stage II may induce the formation of inflorescence axis structures.

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DISSERTATION SUMMARY

Multiple attacks on biological networks

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Due to the general applicability of network models network damage has become a widely examined phenomenon in various fields. Scale-free networks have been shown to be relatively insensitive to random damage however, they are rather vulnerable to attacks targeted to their most-connected elements, called hubs (Albert et al. 2000). In several networks cascading failures may occur and the effects of network topology permanent damage on the resistance of networks have been examined.

Most of the studies used a complete elimination of an element from the network to assess network stability. We would like to provide a general answer to the following question: Is the partial inactivation of several targets more efficient than the complete inactivation of a single target? Using various attack strategies against the *E. coli* (Shen-Orr et al. 2002) and *S. cerevisiae* (Milo et al. 2002) transcriptional regulatory networks we found that partial weakening at a surprisingly small number of points can be more efficient than the complete elimination of a single node.

Robust systems, like the molecular networks of living cells are often resistant to single hits such as those caused by high specificity pharmacons. Here we show that partial weakening of the *E.coli* and *S.cerevisiae* transcriptional regulatory networks at a surprisingly small number (3 to 5) of points can be more efficient than the complete elimination of a single network node. We modeled the networks as directed, weighted graphs and tested a variety attack strategies, such as the elimination of nodes (complete inhibition of proteins), weakening of nodes (partial inhibition of a protein) and weakening or elimination of selected interactions and calculated a decrease in the overall communication efficiency of the network. According to this measure, multiple weak hits provided a similar damage than concentrated attack on one point (Ágoston et al. 2005). For example, the removal of a few, strategically selected interactions in the network are more damaging than removing the best connected node (protein) of the network. These results may help to explain why broad specificity, low affinity pharmacons are often more efficient than their high affinity, high specificity counterparts. Multiple but partial attacks mimic well a number of *in vivo* scenarios and may be useful in the efficient modification of other complex systems.

This and the success stories of multi-target drugs and combinatorial therapies led us to suggest that systematic drug-design strategies should be directed against multiple targets (Csermely et al. 2005). We propose that the final effect of partial, but multiple, drug actions might often surpass that of complete drug action at a single target. The future success of this novel drug-design paradigm will depend not only on a new generation of computer models to dentify the correct multiple targets and their multi-fitting, low-affinity drug candidates but also on more-efficient in vivo testing.

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DISSERTATION SUMMARY

The structural and functional role of phosphatidylglycerol in *Synechococcus* PCC7942 and *Thermoshynecococcus elnogatus* BP-1

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Synechococcus PCC7942 and *Thermoshynecococcus elnogatus* BP-1, cyanobacterial strains are widely used as model organisms for studying the functional and structural aspects of oxygenic photosynthesis. These bacterial strains have a fine structured internal membrane system, the thylakoid membrane system. The thylakoid membrane is the place of oxygenic photosynthesis and has highly conserved lipid composition, which consists of mostly glycolipids and about 10% phosphatidylglycerol (PG) as the only phospholipid. PG has an improtant role in the assembly and maintenance of the photosynthethic apparatus (Hagio et al. 2000). Direct evidence for supression of the electron transfer between QA and QB was obtained by the thermoluminescence and flash-induced fluorescence measurements using a *pgsA* mutant cell line of *Synechocystis* PCC6803 (Gombos et al. 2002). Additionally, it was demonstrated that PG has a primary role in the PSI trimerization process (Domonkos et al. 2003).

In order to investigate the functional role of PG in *Synechococcus* PCC7942 and *Thermosynechococcus elongatus* BP-1 the CDP-dicylglycerol synthase (*CdsA*) gene was chosen for further mutagenesis. CdsA protein catalyzes the first step of the PG synthesis producing CDP-diacylglycerol from phosphatidyc-acid (PA) and CTP. In the PG synthetic pathway there are two other enzymes (*PgsA* and a putaive PGP-phosphatase), that are responsible for the PG, an anionic phospholipid production in a wide variety of bacterial strains (Cronan 2003). Two approaches were used to construct the *ΔcdsA* cell lines. One method was the disruption of the target gene with controlled insertional mutagenesis, and the other technique was used to control the *cdsA* gene expression. Using a controlled gene expression system, the generation of conditionally lethal mutants is much more accessible than that of the null mutants. In our experiments the TetR regulated expression system was used in combination with the $P_{LtetO-1}$ artificial promoter sequence of the pZ vector family, based on *E. coli* studies (Lutz and Bujard 1997). The transformation of cells was carried out by the commonly used transformation method for both strains. The homologous regions were constructed by using the DNA sequence available in public databases in order to guide the recombination-based insertion onto the proper region of the chromosomal DNA. The *ΔcdsA* mutant of *Synechococcus* PCC7942 was segregated and physiologically characterized. The mutant was not able to grow without exogenously added PG to the BG11 medium. In the absence of PG, the mutant lost its viability within 10-11 days of starvation and the cells shape became extremely elongated, suggesting a malfunction of the cell divison process. The oxygen evolving activity of the mutant is drastically decreased after 5-6 days in PG depleted condition simultaneously with a decrease in the cholorophyl/phycobiliprotein ratio. This auxotroph mutant line is suitable for further analysis including DNA chip experiments, and single dye tracking experiments with fluorescent PG.

Using two strains with distinct growth conditions – mesophile and thermophile–, and with specific mutations in them, we can investigate the possible environmental effects on the photosynthetic electron transport chain, the membrane biogenesis, lipid biosynthesis and cell division characteristics.

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DISSERTATION SUMMARY

Characterization the enzymatic activities of the human base excision repair protein Ape2

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The base excision repair (BER) is a very high capacity repair pathway. A lot of DNA damage repaired via this way as miscoding bases, single strand breaks and abasic sites. Only from the abasic sites arise up to 10,000 spontaneously/cell /day.

Class II AP endonucleases are multifunctional enzymes that function in the removal of AP sites as well as 3'-blocking termini. Two families of class II AP endonuclease and repair diesterase enzymes have been identified. The endonuclease IV family contains endonuclease IV of *Escherichia coli* and the Apn1 protein, the major AP endonuclease of *Saccharomyces cerevisiae*. The exonuclease III (exoIII) family includes ExoIII, the major AP endonuclease of *E. coli*, the Apn2 protein of *S. cerevisiae*, the Ape1, the dominant AP endonuclease in human cells and the human Ape2, the newly identified member of this family. ExoIII displays, in addition to the AP endonuclease activity, strong 3'-5' exonuclease, 3'-phosphodiesterase, and 3'-phosphatase activities (Torres-Ramos et al. 2000). Human Ape1, however, has a strong AP endonuclease activity but weak 3'-5' exonuclease, 3'-phosphodiesterase, and 3'-phosphatase activities (Wilson DM 3rd et al. 1995; Suh et al. 1997).

Ape2 is an uncharacterized member of class II AP endonucleases, because it has not been purified yet. Only one recent study reported a weak AP endonucleas activity from partially purified Ape2 fraction. In this study we purified and characterized the Ape2 protein. We expressed the Ape2 protein in a yeast expression system in fusion with a GST-tag. After the purification of the Ape2 protein, we determined the activities of the protein. We were not able to detect any AP endonuclease activity, but we observed 3'-phosphatase activity.

From these experiments we conclude that Ape2 does not have an essential role in the repair of abasic sites, but has a role in the single strand breaks repair. Similar results were published for Apn2, the yeast homolog of the Ape2 (Unk et al. 2000; Unk et al. 2001).

In addition, we demonstrated an 3'-5' exonuclease activity of the Ape2. Our experiments indicated that the 3'-5' exonuclease activity is the major activity of the Ape2.

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DISSERTATION SUMMARY

Oxidative stress tolerance and plant development: the functional characterization of the "oxprot" gene

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Reactive oxygen species (ROS) are toxic compounds produced by normal metabolic processes. Their reactivity with cellular components is a major stress for aerobic cells that results in lipid, protein, and DNA damage. ROS-mediated DNA damage contributes to spontaneous mutagenesis, and cells deficient in repair and protective mechanisms have elevated levels of spontaneous mutations. The human OXR1 (oxidation resistance) gene was described to be involved in the prevention of oxidative DNA damage. OXR1 is a member of a conserved family of genes found in eukaryotes but not in prokaryotes (Volkert et al. 2000). The most highly conserved region of the gene is its carboxyl-terminal half, which contains a TLDc domain (unknown function), furthermore it has a calcium binding EF-hand motif and a mitochondrial localization signal (Volkert and Elliott 2004). Homologues are present in many eukaryotic organisms from yeast to humans, so thus in *Arabidopsis thaliana* (At) and *Medicago truncatula* (Mt) as well. The MtOxprot gene was identified from an alfalfa cDNA library and analized in several ways. It has a relatively high level of expression in the stem, leaf and cell suspension culture as detected by Real-time PCR. Its transcription was increased by various stress treatments as wounding, drought and paraquat (a widely used herbicide generating ROS in plants).

Deletion of the *OXR1* gene in *oxr1* mutant haploid *Saccharomyces cerevisiae* (*scOXR1*) resulted in 10- fold more sensitivity to hydrogen peroxide damage than in wild-type strains. Therefore the MtOxprot cDNA was cloned into a yeast expression vector to check whether it can complement the peroxide sensitivity of the yeast *oxr1_* mutant strain. The results obtained indicates a succesful complementation as the yeast strain expressing the MtOxprot cDNA was approximately 5-fold less sensitive to hydrogen peroxide in a lethality assay than was the *oxr1_* mutant strain.

The human OXR1 gene has two homologues in *Arabidopsis thaliana* (At4g34070, At5g06260), and knock out *Arabidopsis* mutants with T-DNA insertions in the coding regions of the two homologous genes were available (SALK mutants). Neither mutants show hypersensitivity to stress treatments as salinity, paraquat or osmotic stress in seed germination assays, for the possible reason that the homologous genes could complement each other. After crossing the two mutants, double mutant lines will be the subject of further analysis.

Transgenic *Arabidopsis* plants overexpressing the AtOxprot and MtOxprot genes were also generated by an Agrobacterium-mediated in-planta transformation method. Molecular and physiological characterization of the transgenic lines is in progress.

Rice plants overexpressing one of the two AtOxprot cDNA-s (At5g06260; kindly supplied by CropDesign, Gent, Belgium) are also available for the investigations. In preliminary tests the plants showed some kined of tolerance to paraquat treatment indicating that the "oxprot" protein may protect the plant cells from oxidative damage.

In order to produce antibody and characterize biochemically the activity and function of the „oxprot" protein, 6xHis-tagged cDNA clones were overexpressed in *E. coli*, and the proteins were purified from bacterial extracts under native conditions. The *in planta* cellular localization of the oxprot genes will also be determined using fluorescently tagged proteins and the produced antibody.

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DISSERTATION SUMMARY

In situ dissection of the bxd PRE in *Drosophila melanogaster*

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We investigate the regulation of eukaryotic gene expression by the modification of higher order chromatin structure. Our studies focus on the most extensively studied silencer region in the homeotic bithorax complex of *Drosophila melanogaster*, called bithoraxoid Polycomb Response Element (bxd PRE). However, all the previous experiments have used mobile element constructs with various reporter genes; no *in situ* dissection of this region has been performed. In different tests, PREs often proved to be of varying size; moreover, results were sometimes even contradictory. PREs are also known to function cooperatively, and to behave differently in different chromosomal contexts. Therefore, to analyze how PREs function in their natural chromosomal context, and to determine any possible similarities in their structure, we have generated *in situ* deletions within a 3-kb region of the bxd PRE, and examined their effect.

To carry out the deletional analyses of the bxd PRE, we have devised a novel strategy for gene conversion in *Drosophila*. We designed two different types of conversion constructs, which allow us to generate small deletions of either pre-determined or random size. Templates for conversion include FRT-sites flanking small genomic sequences or restriction sites for the yeast I-SceI enzyme, which permit generating small deficiencies by flp/FRT recombination or by the I-SceI induced double strand DNA breaks, respectively. The FRT sites can also be used to merge different deletions generated by us.

Using chromatin immuno-precipitation, 3 sub-elements with potential PRE-activities were identified in the examined 3-kb region of the bxd PRE. However, we have found that only the removal of the central one (670 bp) decreases bxd PRE function to a detectable degree. Flies carrying this deletion showed partial posteriorly directed transformations (wing into haltere, 3rd thoracic segment into 1st abdominal segment) with a penetrance of ~8% when heterozygous. Consistent with our findings, no increase in the penetrance was observed when we extended the size of the deleted sequence to the whole 3-kb region. Considering that bxd and iab-7 PREs share significant similarity in the pattern of GAGA and PHO protein binding sites, we have identified the shortest fragment (184 bp), containing a minimum set of the two protein binding sites, whose removal still lessens bxd PRE function to a detectable degree.

Besides observing adult phenotypes, we have also examined the expression pattern of the Ultrabithorax (Ubx) gene controlled by the bxd region, using antibody staining against the UBX protein in larval CNS. The Gal4VP16 marker gene present in our constructs enabled us to study indirectly the local chromatin structure of the bxd regulatory region through the UAS-GFP system. In agreement with the adult phenotypes, the Ubx gene shows only a modest ectopic expression when the significant part of the bxd PRE is deleted. In contrast to Ubx, GFP shows an extreme ectopic expression pattern even in the head segments. We have also found a striking difference in the gene expression level of these two genes: the intensity of GFP decreased, while it increased in case of Ubx in deletion-bearing heterozygotes. Very likely, these differences reflect the differences between the regulation of the two genes: Gal4VP16 is most likely regulated by a single neighboring enhancer, while Ubx falls under a complex regulation that modifies the effect of bxd PRE deletion. Our data also imply that the bxd PRE binds to at least one Ubx-enhancer and exclusively regulates its activity.

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DISSERTATION SUMMARY

Neurosteroid induced synaptic plasticity in the hypothalamus: the role of the locally synthesized estradiol

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Neuronal plasticity is the remarkable ability of the nervous system to modify the number, morphology and activity of synapses and in such a way the response of a neuron to given inputs. Plastic changes occur both naturally and under experimental conditions, they have been shown to be involved in such phenomena as learning, memory, aging and response to injury. During the last decades it became clear that the gonadal steroids are among those factors, which are able to induce certain adaptive modification of the synaptic connections.

The sex hormones are playing important organizatory role during the development of the nervous system and their action results in sexually dimorphic brain regions. These dimorphic areas differ in size, number of neurons, and synaptic connectivity, all these alterations may serve as a basis for different functioning and for sexually dimorphic behaviour. The hormonal effects, however, are not limited to developmental stages *i.e.* they are influencing the plastic changes in the adult brain, too.

In female rodents a continuous cycling synaptic remodeling is taking place in the hypothalamus that are linked to hormonal variations during the ovarian cycle. Experimental data show that the synapse remodeling in the hypothalamic arcuate nucleus is driven by 17ß-estradiol, because in ovariectomized rats the hormone substitution resulted in reversible decline of synapses. It has also been demonstrated that the hormonally induced changes of axo-somatic inputs to arcuate neurons is specific, because not all of the synapses are affected. To better understand the hormonally induced synaptic remodeling, it would be necessary to describe the changes in the number and/or in the structure of synapses in different areas of the nervous system.

In the present study the anteroventral periventricular nucleus (AvPv) of adult rats was chosen for analysis, due to its abundant estrogen- and progesterone-receptive neurons and its critical role in the control of gonadotrophin secretion.

12 female rats were ovariectomized (OVX) at age of 2 months and sacrificed 4 weeks later. Six animals were injected sc. with 17ß-estradiol (100g / 100g body weight), the rest was used as controls injected with sesame oil.

The combination of pre- and postembedding immunostaining was used to investigate the synaptic connections of estrogen receptor-immunoreactive (ER-ir) and non-ER-ir neurons in the AvPv.

Ultrastructural analysis revealed that the AvPv neurons of OVX animals receive approximately the same number of GABA-immunoreactive (inhibitory) and non-immunoreactive (probably excitatory) axo-somatic synapses. In contrast with the arcuate nucleus, 17ß-estradiol treatment of OVX rats did not result in changes of GABAergic axo-somatic synapses, but we observed a significant increase of non-GABAergic contacts and a decrease of all types of axo-dendritic synapses. The innervation patterns of ER-ir and non-ER-ir neurons were different.

To study the hypothesis that the locally synthesized estradiol has an effect on synaptic connectivity we treated the animals with the precursor of 17ß-estradiol dehydroepiandrosterone (DHEA). This neurosteroid could also induce changes in AvPv synapses, but its effect was blocked by the aromatase inhibitor letrozol. This observation suggests that the conversion of DHEA into estradiol is involved in the mechanism of action of this neurosteroid on synaptic remodeling.

Our data indicate that 17ß-estradiol induces synaptic remodeling in the AvPv nucleus and may play a decisive role in the regulation of gonadotrophin secretion. On the other hand the present study shows that the molecular mechanisms responsible for the hormonally induced synaptic remodeling in AvPv and arcuate nucleus are different, which may reflect the fact that these two nuclei are responsible for different functions.

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DISSERTATION SUMMARY

Isolation and characterization of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) of *Rhizomucor miehei*

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Rhizomucor miehei is a practically and theoretically important member of the genus *Rhizomucor* (order *Mucorales*). Certain strains of this genus are used in the food industry, and some isolates are agents of opportunistic infections in man and animals. The genus *Rhizomucor* involves two ubiquitous species: *R. pusillus* and *R. miehei*. The purpose of our study was to clone and characterise the coding and regulatory region of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) gene of *R. miehei*, a homothallic species of the genus. HMG-CoA reductase (EC 1.1.1.34) catalyses the reduction of HMG-CoA to mevalonate, which is the first step of the acetate/mevalonate pathway, leading among others to the synthesis of the characteristic mating pheromone (trisporic acid) of zygomycetes.

Degenerated primers designed to the most conserved region of known *hmgr* genes were used to amplify a short conserved region of the gene by polymerase chain reaction. The resulting 314 bp length DNA segment of the *hmg* R gene was labelled with non-radioactive dioxigenine and was used as a homologous probe to select positive clones from a genomic library prepared by Vastag et al. (2004) from the strain *Rhizomucor miehei* (NRRL 5901) in λ FixII phage. One λ phage clone was selected and, after purification steps, it was revealed that this clone contains an insert of about 8000 bp. This insert was cloned into pBluescript plasmid with *Xho*I restriction endonuclease and was used for further subcloning and sequencing experiments. As a result of these experiments, the complete nucleotide sequence was determined and analysed. The putative protein sequence proved to be 1059 amino acids in length. Five introns have been identified in the HMG-CoA reductase gene, dispersed in the whole coding region. Two types of method were carried out in order to determine the *hmgr* gene copy number of *R. miehei*: quantitative PCR and Southern blot experiments revealed that both *R. miehei* and *R. pusillus* have two *hmgr* genes. In order to prove that the cloned *hmgr* gene of *R.miehei* is expressed, total RNA was isolated and reverse transcription was carried out with specific primers.

The sensitivity to lovastatin, a competitive inhibitor of HMG-CoA reductase, was determined in the two *Rhizomucor* species, in order to investigate if the *hmgr* gene could be utilised in transformational experiments by providing lovastatin resistance to the transformants (Vágvölgyi et al. 2004). It was observed that *R. pusillus* isolates are more susceptible to lovastatin than *R. miehei*, despite both having two copies of the *hmgr* gene. The difference in the susceptibilities of *R. pusillus* and *R. miehei* is so marked that besides the isoenzyme (Vastag et al. 1998) and random amplified polymorphic DNA (RAPD) patterns (Vastag et al. 2000), it could be used for differentiation of the two *Rhizomucor* species. Accordingly, a simple and reliable method for species-level differentiation of these species (Lukács et al. 2004) has been devised.

In order to broaden the available expression data on the *R. miehei hmgr* gene, further experiments are in progress.

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DISSERTATION SUMMARY

The novel *Drosophila* formin dDAM regulates the actin cytoskeleton in the tracheal system and the CNS

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Proteins of the formin family plays key roles in the regulation of the actin cytoskeleton. They can nucleate new actin filaments directly, and regulate the polymerization of the growing actin bundle. The formins are multidomain proteins containing several important homology domains: the FH1, FH2, and FH3 formin homology domains, an N-terminal RBD domain, and a C-terminal DAD autoinhibitory domain. The FH2 domain has been shown to be both necessary and sufficient for actin nucleation and polymerization *in vitro*, the FH1 domain is able to bind the G-actin-binding protein profilin, while the FH3 domain has been implicated in the regulation of the subcellular localization of the protein (Evangelista et al. 2003). The largest subclass of formins, called diaphanous-related-formins (DRFs) are activated upon Rho-GTP binding to their RBD domain. This binding alleviates the intramolecular autoinhibitory interaction between the RBD and the DAD domain (Evangelista et al. 2003).

Recent studies have led to the identification of a novel subtype of formins, DAAM that has been implicated in planar signaling during *Xenopus* gastrulation. These results suggested that DAAM might function as a bridging factor between the signaling molecules Dsh and RhoA because Daam1 binds to both Dsh and RhoA, and Wnt/Fz activation of RhoA depends on Dvl (a Dsh homologue) and Daam1 (Habas et al. 2001). However, contrasting to this model, much of previous work provided evidences that formins are Rho effectors that act downstream of the Rho GTPases.

To begin the genetic analysis of *dDAM*, first we have isolated a set of *dDAM* mutant alleles by P-element excisions. We found evidences that *dDAM* is involved in the regulation of the actin cytoskeleton in several different tissues. Below we consider the trachea and the CNS function of *dDAM*.

During the first phase of tracheal development the primordial cells invaginate from the epidermis and form the primary branches. Subsequently, some tracheal branches fuse with an adjacent branch to build up a continuous tubular network. Finally, tracheal cells secrete a cuticle on their apical surface that protects the larvae from dehydration. Interestingly, the tracheal cuticle is distinguished from the epidermal cuticle by the presence of cuticle ridges often called taenidial folds. In the absence of *dDAM*, the taenidial folds fail to organize into parallel running cuticle ridges leading to the collapse of the tube. Our results demonstrate that in tracheal cells apical actin is also organized into parallel running rings just as the overlaying cuticle ridges, and *dDAM* severely impairs actin organization. Taking it together, it appears that *dDAM* directs cuticle secretion in the tracheal system by polymerizing and organizing apical actin into parallel running bundles.

Additionally, we have analysed the CNS function of this formin and found that *dDAM* is required for axon elongation in the embryonic CNS. Interestingly, the vertebrate homologues of *dDAM* are also expressed in the CNS raising the possibility that the regulation of axon growth is an evolutionary conserved function of the DAAM subfamily of formins.

In order to understand the regulation of *dDAM*, we have identified genetic ineractors and collected evidences that suggest that *dDAM* acts together with *RhoA*, and two members of the SRC family of kinases to regulate the actin cytoskeleton in the trachea.

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DISSERTATION SUMMARY

The origin of lamellocytes in *Drosophila melanogaster*

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Drosophila larvae defend themselves with a sophisticated cellular and humoral immune system, which shares similarities with the innate immune system of vertebrates. Therefore *Drosophila* became an excellent tool to study the innate immune reactions and host-parasite relationship (Hultmark 2003) in general. The cellular components of the *Drosophila* immune system are the hemocytes, they are present in three hemocyte compartments: in the circulation, in the lymph glands and attached to the cuticle, the latter are being the so called sessile hemocytes. In the larval circulation there are three differentiated cell types: plasmatocytes, lamellocytes and crystal cells. The majority (99%) of the hemocytes are plasmatocytes; they are responsible for phagocytosis of bacteria and apoptotic cells. The lamellocytes are large flattened cells, seldom seen prior to pupariation in wild type larvae. Upon parasitic wasp infestation the number of lamellocytes is dramatically increased in the circulation; their role is to eliminate foreign bodies and abnormally developing tissues by encapsulation (Rizki 1984). It is believed that following wasp infestation the lamellocytes originate from the lymph glands (Lanot et al. 2001; Sorrentino et al. 2002). Our preliminary data however suggested that this can not be the case.

We have searched for the origin of lamellocytes in different hemocyte compartments in the larva by infesting the larvae of the Hemese-Gal4 and Hdc transgenic flies. The hemocyte compartments of these larvae can be caracterized by the expression pattern of the *Hemese*-Gal4 driver (Zettervall et al. 2004) and the *headcase* gene (Weaver and White 1995). The presence of the *Hemese*-Gal4 and the absence of the *headcase* is specific to hemocytes from circulation and sessile tissue, while the absence of Hemese-Gal4 and expression of *headcase* is characteristic to the lymph gland hemocytes. We have correlated these compartment specific and the lamellocyte specific markers to define the origin of lamellocytes.

Following wasp infestation the differentiating lamellocytes in the circulation had the same phenotype as the lamellocytes found in the sessile tissue, thus we conclude that lamellocytes originate from the sessile tissue.

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DISSERTATION SUMMARY

Vegetative incompatibility in *Aspergillus niger*

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The parasexual cycle, recombination by cell fusion and subsequent mitotic recombination, is a well known phenomenon in ascomycete fungi. Beside the sexual cycle, this is an alternative way for genetical recombination between isolates. For fungal strains which do not have a sexual life cycle, this is the only way for recombination.

The process of cell fusion is controlled by the so called *het* (heterokaryon incompatibility) or *vic* (vegetative incompatibility) genes. The number of *het* genes differs in every genus. If there is at least one *het* locus which differs in the strains forming anastomoses, karyogamy (and in certain strains even plasmogamy) does not occur and autolytical processes are induced. This process is an effective barrier against infectious genetic elements, like mycoviruses or plasmids and can prevent resource plundering. The incompatibility systems can be allelic or non-allelic; in the first case different alleles of the same gene, while in the latter case different genes rule the process, respectively. In certain strains only one of the systems work.

Aspergillus niger is a member of a species-complex with the same name. It became an increasingly important microorganism in the last few decades, especially due to industrial applications. Mapping of its genome was finished in 2001 and now it is available for scientists. The fungus has a unique life-cycle: it does not appear to have a sexual cycle and most of the natural isolates are highly incompatible with each other. Possibilities for genetic recombination thus seem to be highly reduced in *A. niger*. We aimed to get a better understanding of the incompatibility process and the genes regulating the steps. There is a worldwide collection of wild *A. niger* isolates and many of mutant strains which are available for use in our experiments.

het-c is one of the well characterized incompatibility genes. It has one known allele in *Podospora anserina* and in *Aspergillus nidulans* and three alleles in *Neurospora crassa* (termed as Groveland, Oakridge and Panama; these allels differ in a 50 amino acids long insertion/deletion motif). Although the *het-c* homolog of *P. anserina* is not allelic, the *het-c*^{Panama} allele of *N. crassa* could trigger incompatibility reaction.

Based on PCR experiments we established that the *het-c* gene from different *A. niger* isolates does not have an allelical region (this feature differs from *N. crassa het-c*). On the other hand different isolates bear different numbers of glutamine-repeats at the C-carboxy terminus of the protein. The role of the glutamine-repeats has not cleared yet. In protoplast-transformation experiments the *het-c*^{Panama} allele of *N. crassa* triggered an incompatibility reaction, similarly to that observed in *P. anserina*.

In the near future we plan to make some protoplast-transformation experiments with the modified *het-c* of *A. niger* and GFP-tagged (green fluorescent protein) *N. crassa het-c* alleles.

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DISSERTATION SUMMARY

Adaptive responses to high salinity of two subspecies of *Aster tripolium* on different nitrogen sources

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Soil salinity is an important agricultural problem. One possible way to use affected fields is planting salt tolerant crops such as *Aster tripolium* L.

Aster tripolium is a typical halophyte species with two horizontally isolated subspecies (Borhidi 1995). The ssp. tripolium is a maritime halophyte, while ssp. pannonicus is common on the continental alkaline salty meadows rich in $NaHCO_3$. The two subspecies are different concerning their habitats, morphology and physiology. Both ssp. accumulate inorganic ions (especially Na^+) even at low external concentrations. Physiologically, sea aster was more intensively studied (Shennan et al. 1987ab) because of its recently increasing commercial importance as halophyte crop.

In our experiments, adaptive responses to high salinity of two subspecies of *Aster tripolium* were studied at different pH values and Na^+ concentrations and on different nitrogen sources.

Plants were grown hydroponically in complete modified Hoagland nutrient solution of different pH values (from 4 to 10). On the basis of protein content data, pH 5 and 8 were selected for further experiments when plants were grown at 0, 50, 100, 200 and 300 mM NaCl concentrations added to the complete nutrient solution. Chlorophyll, protein content and cation concentrations were measured. Ssp. tripolium obviously suffered under low salt conditions combined with high pH values as shown by the low pigment concentrations. In both subspecies, qualitative and quantitative alterations were observed in protein concentrations with increasing salinity and pH values. At low pH and medium salt concentrations, ssp. tripolium had higher protein levels than ssp. pannonicus, while at high pH values ssp. pannonicus had higher performance. Very high Na^+ concentrations were accumulated in the leaves in both pH regions. Calcium is known to play a special role in tolerance under salinity. Surprisingly, in ssp. pannonicus Ca^{2+} accumulation increased under the highest NaCl concentration (300 mM), while in contrast, ssp. tripolium showed a decreasing tendency in calcium accumulation under increasing external salinity (Sági and Erdei 2002).

In the following, our intention was to investigate enzymes involved in nitrogen metabolism as a function of Na^+ concentration. We selected pH 5 and nitrate or ammonium as nitrogen source at 0, 10, 50, 100, 200 mM NaCl in the nutrient solutions. Ion concentrations (Na, Fe, K, Ca and Mg), nitrate reductase (NR) and glutamine synthetase (GS) activity were measured in both leaves and roots. One of our interesting result was that young leaves of *Aster tripolium* ssp. tripolium treated with NH_4^+ and relatively low NaCl concentrations, became chlorotic, while on high salt concentrations the signs of NH_4^+ toxicity was observed (toxicity symptoms: roots turn brown and appear unhealthy, with necrotic root tips; plant growth is decreased; necrotic lesions occur on stems and leaves).

In plants, grown on solution containing NH_4^+, in the root of both subspecies (but more accented in ssp. pannonicus) GS activity increased as a function of Na^+ concentration. In the leaf, GS activity was also enhanced. Data suggest that when the nitrogen source was nitrate, the scene of nitrate processing was not in the root, since GS activity was about threefold higher in leaves than in roots, and it was much lower than in the roots of plants grown on NH_4^+. It seems that ammonium uptake is enhanced by NaCl. This result was also supported by the NR activity data. In plants grown on NH_4^+, NR activity decreased with increasing NaCl concentrations in both subspecies (it is more distinct in ssp. tripolium). This can be a reason of the abundancy of NH_4^+ at high Na^+ concentrations. Similarly to GS, in plants grown on nitrate, NR activity was also higher in leaves than in roots and showed higher values than in plants grown on NH_4^+.

We conclude that adaptive responses, including changes in nitrogen metabolism, are different under salt stressed circumstances in the two subspecies.

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DISSERTATION SUMMARY

Echophysiological and molecular investigation of *Trichoderma* strains isolated from winter wheat rhizosphere

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There is a worldwide need to adopt the practice of sustainable agriculture, using strategies that are environment-friendly, less dependent on agricultural chemicals and less damaging to soil and water resources. One of the key elements of such sustainable agriculture is the application of biocontrol agents for plant protection. Species in the filamentous fungal genus *Trichoderma* are of great economic importance as sources of enzymes and antibiotics; plant growth promoters; degraders of xenobiotics, and most importantly, as commercial biofungicides (Howell 2003), thus they are potential candidates for biocontrol applications.

One hundred and twenty *Trichoderma* strains were isolated from roots of winter wheat grown in agricultural fields of southern Hungary from different defined test holes. The identity and diversity of species was examined based on morphological, biochemical and molecular characters. The morphological data were collected by measuring structure and shape of conidiophores, phialides and conidia. Differential utilization of a total of 100 carbon and 45 nitrogen sources were applied as biochemical markers. For the investigation of molecular diversity, sequence analysis of the internal transcribed spacer (ITS) region and cellulose-acetate electrophoresis (CAE) mediated isoenzyme analysis were applied. In the case of CAE, after initial testing of 13 enzymes for activity and resolution of bands, 7 of them (glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, 6-phosphogluconate dehydrogenase, peptidase A, B, D and phosphoglucomutase) proved to be appropriate for the analysis of the full sample set. Comparing the different electrophoretic types, each of the enzymes applied could be used as molecular marker in the identification of *Trichoderma* spp (Szekeres et al. 2004a). The differential utilization of carbon and nitrogen sources, the sequenced ITS regions and the isozyme data of the isolates were used for the construction of a phylogenetic dendrogram.

The isolated strains were investigated for the production of β-xylosidase, α-glucosidase, β-glucosidase, β-galactosidase, cellobiohydrolase, trypsin-, chymotrypsin- and chymoelastase-like proteases and *N*-acetyl-β-glucosaminidase, which are extracellular enzymes important for the biocontrol activity. The secretion of enzymes was revealed by means of specific chromogenic *p*-nitroanilide and *p*-nitrophenyl substrates. Some of the examined enzymes were secreted constitutively and their amounts showed high variability within the isolates.

Biocontrol properties were tested *in vitro* against *Fusarium culmorum* NRRL 29371, a significant pathogen of wheat. Direct confrontation assay was applied for recording the inhibition effect, which was expressed as the value of biocontrol index (BCI) calculated from the image analysis of ratio of the area occupied by *Trichoderma* and the plant pathogen. A modified plant pathogen aggressiveness test was carried out on wheat seeds for the investigation of reduction in diseases severity in the presence of *Trichoderma* strains.

After collecting the taxonomical and ecophysiological data, the relationships and correlations between the biocontrol efficiencies, the productions of several extracellular enzymes and location of isolation were examined by statistical analysis. To improve the antagonistic capacity of a selected fungal strain, a mutagenetic program was undertaken for the construction of derivatives overproducing extracellular proteases. The mutant strains were obtained by means of UV-irradiation and were selected for *p*-fluorophenyl-alanine resistance or altered colony morphology. Both trypsin-like and chymotrypsin-like protease secretion was elevated in most of the mutant strains. The profiles of protease isoenzymes were different between the mutants and the wild-type strain, when examined by gel filtration chromatography. Certain mutants proved to be better antagonists against plant pathogens in *in vitro* antagonism experiments (Szekeres et al. 2004b).

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DISSERTATION SUMMARY

Developing a novel method to identify genes involved in germ line induction of *Drosophila melanogaster* embryos

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Embryonic germ cell development in *Drosophila melanogaster* depends on the germ plasm, the most posterior part of the ooplasm. There are experimental evidences that germ plasm contains all the factors necessary for the formation of the embryonic germ cells (Mahowald 2001). Though most of the germ plasm components have not been identified yet, it is known that at least in part the germ line factors are stored in the form of RNA (Tomancak et al. 2002). The aim of our work was to identify novel germ plasm-enriched, localized RNAs by combining cDNA microarray and RNA *in situ* hybridization techniques and to verify the results with a functional analysis.

In the cDNA microarray experiments we compared deficient, normal and ectopic germ plasm conditions using a chip containing 3200 cDNAs of annotated *Drosophila* genes. Sixty RNA species were selected as exhibiting the expected microarray pattern and subsequently investigated on their distribution in wild type and ectopic germ plasm containing embryos. We found that 17 out of 60 showed germ plasm specific localization (Szuperák et al. 2005).

For the functional analysis of the localised RNAs and to identify additional germ plasm specific RNAs we have established a genetic interaction type of assay. This assay is based on a strain that carries three mutations in heterozygous form resulting in a moderate germ cell deficient phenotype. Using this sensitized genetic background we performed a screen on a third chromosomal P-element insertion collection. 600 such mutations were analysed and 26 of them were significant enhancers of the germ cell deficient phenotype. The site of the P-element insertions in these 26 lines were molecularly mapped and the affected genes were identified by BLAST search. The BLAST searches revealed that one of the 26 lines were selected previously in the microarray experiment and some of them turned out to be alleles of genes whose function in the germ line development has been already established.

These results indicate that we have developed a reverse genetic experimental system which in combination with the genetic interaction assay enables the isolation at genom scale level and the analysis of new factors involved in Drosophila germ line differentiation.

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DISSERTATION SUMMARY

Using labeled mutant cytochromes for the examination of intraprotein electron transfer

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One of our purposes is to investigate the role of the protein matrix in electron transfer processes. To this end we measure the elecron transfer kinetics spectroscopically on various cytochromes of the mitochondrial respiratory chain.

To initiate electron transfer in cytochrome c we use a photoactive protein label, 8-thiouredopyrene-1,3,6-trisulfonate (TUPS) (Kotlyar et al. 1997). TUPS has two slightly different chemical forms, one can label surface lysines and the other one cysteines. Upon light excitation the triplet state of the dye forms with high quantum efficiency. The triplet has a long lifetime (0,5 ms) and low redox potential, therefore it is a strong reductant, capable of donating its electron to a variety of acceptors. We have shown that TUPS is also capable of acting as a photoactive electron acceptor, depending on the redox partner (Kotlyar et al. 2004).

The selected cytochrome c for electron transfer measurements is the horse heart protein, because it contains only two cysteine amino acids, which are inaccessible, since they participate in heme binding. Therefore single cysteines introduced on the surface of the protein allow exclusive labeling by TUPS and the initiation of electron transfer from different positions of the protein. In c-type cytochromes covalent heme binding is catalysed by the enzyme heme lyase during cytochrome c maturation. For that reason cytochrome c gene expression was performed together with the heme lyase gene. For expression of the cytochrome c and heme lyase genes we used the pBAD24 plasmid (Guzman et al. 1995), a member of a vector family containing the inducible and controllable powerful P_{BAD} promoter of the arabinose operon (*araBAD*). The proteins were heterologously expressed in BL21(DE3) strain of *E. coli*.

We have measured electron transfer on cytochrome c labelled on the K8 and K39 side chains, positioned at opposite sides relative to the heme cofactor, as well as on K8C and K39C introduced by site directed mutagenesis. These changes allow us to compare the electron transfer rates when the lysines are replaced by cysteines, thereby altering both the chemical nature and the length of the putative pathway between the electron donor and acceptor.

The experiments show us that the electron transfer between TUPS and the heme of cytochrome c deviates from the expected monoexponential kinetic behavior. Neither the overall rate, nor the individual exponential components of electron transfer, as followed by kinetic absorption spectroscopy, correlate with the length of the covalent link connecting the dye with the protein. Molecular dynamics calculations show that TUPS can approach the protein surface and occupy several such positions. This heterogeneity may explain the multiexponential electron transfer kinetics. The calculated optimal electron transfer pathways do not follow the covalent link but involve through space jumps from the dye to the protein moiety, effectively decoupling the length of the covalent link and the electron transfer rates (Tenger et al. 2005).

Our further goal is to measure electron transfer in the complex of cytochrome c and cytochrome c oxidase. In order to optimize the reduction of cytochrome oxidase, we were searching for the optimal label position on the surface of cytochrome c. We expressed the G1C, V3C, V11C, A15C, G23C and G34C mutants, and our preliminary measurements indicate that two of them (V11C and A15C) are fast enough to initiate efficient electron transfer in cytochrome c oxidase. This will enable us to investigate the details of the electron transfer route and the catalitic activity of cytochrome oxidase.

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DISSERTATION SUMMARY

Transcriptional targets of *Drosophila* p53

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The tumor suppressor p53 is a sequence specific transcription factor which plays a crucial role in mammalian cells in safeguarding the integrity of the genome. The p53 protein regulates multiple cellular responses to DNA damage, including DNA repair, induction of apoptosis, cell cycle arrest, but the transcriptional targets that specify these processes are mostly unknown.

The *Drosophila* homolog of p53 (Dmp53) has been identified recently (Ollman et al. 2000). Dmp53 is required for the apoptotic response, but unlike mammalian p53, it appears unable to block the cell cycle in G1 phase (Ollman et al. 2000). Since in many aspects Dmp53 has similar function as its human counterpart, studying p53-associated functions in the genetically well traceable *Drosophila* system offers many advantages. In concert with this, the aim of my work is the identification of Dmp53 transcriptional targets activated upon different cellular stress responses. For this I use UV-C and X-ray to induce genotoxic stress and study the change of gene expression pattern between treated and untreated animals. After the optimalization of the dosage and the recovery time of UV-C and X-ray irradiation, I compared the expression of *Ark*, *Hid* and *reaper* genes, which are known targets of Dmp53 (Jassim et al. 2003; Brodsky et al. 2000), in third instar larvae by real time PCR. I observed that X-ray irradiation resulted in *reaper* induction, while the level of *Ark* mRNA was enhanced upon UV-C irradiation. *Hid* expression was upregulated under both stress conditions used. None of these genes showed altered expression in *Dmp53* null mutants, indicating that their expression was indeed the result of Dmp53 activation. As these observations indicated that my experimental system is suitable for the identification of Dmp53 regulated genes, I carried out genome-wide studies using DNA microarrays to compare the gene expression profile of UV-induced and non-induced animals in wild type and Dmp53 mutant background. In the microarray experiment I observed 29 transcripts upregulated and 38 transcripts downregulated upon UV-C irradiation. The expression of these genes remained unchanged in *Dmp53* null mutant animals suggesting that Dmp53 participates in the regulation of their transcription. One of these genes is an ubiquitin ligase with homology to the mammalian MDM2 and MDM4 proteins, which are the main regulators of p53 (Momand et al. 1992; Oren 1999). MDM2 is a transcriptional target of p53 and regulates the level of p53 protein in the „p53-MDM2 loop" (Oren 1999) A similar regulation of Dmp53 is unknown so far, therefore further studies of the identified MDM2-related protein might result in significant new findings in the mechanisms p53 exerts its many effects.

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OBITUARY

Dr. Ferenc Kevei (1942-2003)

Ferenc Kevei, teacher, researcher, inspiring personality and friend to many, passed away on 27 September 2003. His life as a scientist spanned over almost 40 years, and was dominated by his love for fungi, a love carried over to many of his students, colleagues and collaborators.

He was born on 29 September 1942 in Kőszeg, where he attended school (1948-1960). Between 1960 and 1965 he studied at Attila József University in Szeged, and was awarded his diploma in 1965 as a teacher of biology and chemistry. Following this he worked at the Department of Plant Physiology of Attila József University until 1972 when he joined the staff of the Department of Microbiology. From 1998 till 2003 he was head of this department. He carried out enormous teaching activities, holding lectures, seminars and laboratory practicals and supervizing research projects for graduate and PhD students in microbiology. He took an active part in the organization of the new graduate and postgraduate programmes at the university. At the beginning of his academic career his research was focused on the mode of action of steroid glycosides with antifungal activity. This was the topic of his doctoral thesis. At that time he started to work with fungal protoplasts. He additionally studied the possibility of gene transfer among filamentous fungi, especially *Aspergilli*. His thesis entitled "Protoplast fusion of *Aspergilli*", was defended in 1981. He became a Candidate of Biological Sciences in 1981. During the last two decades his interest turned to the mitochondrial DNA organisation of "black *Aspergilli*". On the basis of the work of his team the species of *A. niger*, *A. tubingensis*, *A. carbonarius* and *A. japonicus* were divided into several subgroups according to the RFLP patterns of their mitochondrial DNA. They determined the whole nucleotide sequence of the mtDNA of one representative of both *A. niger* and *A. tubingensis*. Using the protoplast fusion technique, they successfully carried out mitochondrial DNA transmission among heterokaryon-incompatible *Aspergillus* strains. They verified the role of mobile introns in the formation of recombinant progenies. He defended his habilitation in 1999, completed his doctoral thesis in 2002 and became a Doctor of Sciences in 2003. His role in higher education and scientific works were awarded by many honours, among others by the Manninger Rezső Medal of the Hungarian Microbiological Society (2001), and the Széchenyi István Professorship established by the Ministry of Education (1997-2000 and 2001-2003). He was a member of the Society for General Microbiology, the British Mycological Society, the Hungarian Microbiological Society, the Society Hungarian Geneticists, the Hungarian Biological Society, the Hungarian Biochemical Society and the Committee of General Microbiology of the Hungarian Academy of Sciences.

Feri was active right up to the last second of his life, working in the laboratory at the Department with his much-loved *Aspergilli*, writing manuscripts, organizing the university life, travelling and attending conferences. Learning and his quest for discovery never stopped. He inspired and enriched the lives of so many.

Selected publications

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OBITUARY

Dr. Lajos Ferenczy (1930-2004)

Prof. Dr. Lajos Ferenczy, one of the most foremost microbiologists in Hungary and a member of the Hungarian Academy of Sciences, died on19 March 2004 following a short illness. As Head of the Department of Microbiology and later the Microbiology Research Group of the Hungarian Academy of Sciences at the University of Szeged, he established an excellent scientific school. As a scientist, he conducted pioneering research on fungal protoplast fusion, a revolutionary technique in biotechnology. He was a famous and highly-regarded scientist on an international scale.

He was born on 25 October 1930 in Kisújszállás. He attended the Faculty of Sciences at the University of Szeged in 1949. During the period of his undergraduate studies, he won a Fellowship from the Hungarian Academy of Sciences and wrote 3 award-winning papers. One of them was later published in Nature (Ferenczy L (1956) Antibacterial substances in seeds. Nature 178: 639-640). In 1953, he graduated in biology and chemistry at the University of Szeged, where, in the same year, he obtained a position at the Department of Plant Physiology. In 1958, he was awarded his university doctorate at the University of Szeged and in 1960 he received a C.Sc. (corresponding today to a Ph.D.) from the Hungarian Academy of Sciences. In 1980, he received his D.Sc. from Hungarian Academy of Sciences for his thesis entitled "Gene Transfer via Protoplast Fusion in Fungi".

He was a respected teacher at the University of Szeged from the very first steps of his career until his death. From 1953 to 1954, he worked as Teaching Assistant, from 1954 to 1962 as Research Assistant, from 1962 to 1964 as Assistant Professor, and from 1964 to 1981 as Associate Professor. He became a Professor in 1981. The Department of Microbiology at the University of Szeged became an independent department mainly as a result of his enthusiastic work. He was Head of this Department from 1972 to 1997.

In 1969 and 1970 he was a Visiting Associate Professor at the Department of Plant Pathology at the University of Illinois, Urbana-Champaign, USA, working together with Prof. David Gottlieb. From 1987 to 1989, he was a Visiting Professor at the Institute of Microbiology, Federal Institute of Technology, in Zürich, Switzerland.

Between 1998 and 2000, he was Head of the Microbiology Research Group of the Hungarian Academy of Sciences at the University of Szeged. From 2000 until his death, he was Research Professor of the Hungarian Academy of Sciences.

At the University of Szeged he pursued different social activities. From 1972 up to his death, he was a member of the Biology Committee and of the Habilitation Committee of the Faculty Council. He was active in the Senate of the University and as President of the Ph.D. Committee for Molecular Biology and Biotechnology. From 2000, he was a member of the Science Committee of the Faculty of Sciences. For his outstanding education at Hungarian Academy of Sciences and workshop funding activity, he was honoured by various organizations.

In 1987, Lajos Ferenczy was elected a Corresponding Member of the Hungarian Academy of Sciences. From 1995, he was an Ordinary Member. He took an active part in shaping the science policy as vice president of the Biology Section of Hungarian Academy of Sciences, chairman of the General Microbiology Committee, president of the Organizing Committee for Life Sciences and a member of the Hungarian EMBO Committee and Qualification Committee.

In recognition of his rich scientific achievements, Lajos Ferenczy was granted numerous high awards by the Hungarian Academy of Sciences, the Hungarian Microbiological Society, the Hungarian Ministry of Education, the Hungarian Minister of Health and the Hungarian Government. He received the Purkyne Medal at the University of Brno in 1981. He was elected a member of various foreign scientific societies, the most outstanding among them being the Academia Europaea (Cambridge, England), the American Academy of Microbiology and the US National Academy of Sciences.

During his last 35 years, Professor Ferenczy was highly active in many international training courses, symposia and

conferences as organizer, keynote speaker, demonstrator, chairman and editor of symposium proceedings in the fields of protoplast fusion, fungal gene transfer, somatic hybridization and genetic engineering. He was the main organizer and president of the First Hungarian Mycological Conference at the Hungarian Academy of Sciences in Budapest in 1999. Generations of mycologists grew up under his mentorship. His death is a tremendous loss for the mycological world. We shall remember him with our greatest respect.

From among Lajos Ferenczy's major communications

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OBITUARY

Dr. Á. Magdolna Gulyás (1948-2005)

Magdolna Gulyás was born on 9th October 1948 in Szeged. Following her schooling in Szeged, she entered the Faculty of Science at József Attila University, from where she received her diploma in biology in 1972. After graduating, she started work as a biologist at the Animal Health Station of Csongrad County.

In 1973-74 years she worked at the Department of Biophysics of József Attila University, where she prepared her doctoral dissertation on a biophysical topic: „Study of the spectroscopic properties and transfer of electron excitation energy in a fluorescent lysozyme and fluorescein isothiocyanate complex system". She was awarded her doctorate in 1976 with the result „summa cum laude".

From 1974, following the foundation of the Department of Biochemistry at József Attila University, she worked there as an assistant and then a senior lecturer. She was dedicated to her work, which involved the study of the properties and stability of immobilized enzymes. She defended her dissertation for the candidate degree: „The heat stability of immobilized enzymes and some applications" in 1992, and received the title of Candidate of Biological Science from the Hungarian Academy of Sciences. In the next year she was appointed associate professor. Between 1994 and 2002 she was head of the Department of Biochemistry. Her scientific activities included the study of stress responses of environmental pollution, e.g. pesticides, herbicides and heavy metals in fish. She was especially interested in investigations of enzyme systems participating in antioxidative processes. She paid great attention to proteins involved in the first line of biological defence (metallothioneins and heat shock proteins). Her successful scientific achievements are revealed by numerous publications in various prominent, international journals and she was widely recognized for her work on environmental toxicology. She created new, special subjects and introduced the teaching of environmental biochemistry and stress biochemistry for students. She was co-author of a scientific book and textbooks. She always paid detailed attention to the education of the students. Many students learned from her and four PhD dissertations were defended under her guidance.

In 1997 Magdolna and her research group were awarded the Ibaraki Kasumigaura Prize for their invaluable contributions to the success and achievement of the 7th International Conference on Lake Conservation and Management in San Martin de Los Andes (Argentina).

Besides her love for research and teaching, she dedicated a great deal of her time to serving the scientific community. She was a founder and active member of the Environmental Biochemical Section of the Hungarian Biochemical Society. She was a member of the International Society of Ecotoxicology and Environmental Safety and the Hungarian Biochemical and Toxicological Society.

She was full of scientific plans when she was taken away from us extremely suddenly on 10th March 2005 after some days in the university hospital.

She was a devoted, exemplary mother. Her early death means that she could love and take pleasure in her granddaughter for only a short time.

Her death is a great loss to the scientific community at large. We miss her terribly. Her colleagues, and her students at the Department of Biochemistry consider it an enormous privilege to have had the opportunity to work with her and we are deeply grateful for her presence among us. We should like to preserve her spirit and we shall all remember her with great affection.

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References

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Bloom FE (1983) The endorphins: a growing family of pharmacologically pertinent peptides. Annu Rev Pharmacol Toxicol 23:151-170.

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